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Influence of Weight Loss on Metabolic and Mitochondrial Function in the Mature Horse

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ABSTRACT

Alterations of Metabolic and Mitochondrial Function in Response to Weight Loss

Jennie L. Zambito

Obesity causes a multitude of metabolic issues in the horse, yet stepwise alterations in glucose and lipid metabolism, mitochondrial capacity and oxidant status during weight loss have yet to be analyzed. Skeletal muscle contains subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria, which respond differently to physiological stimuli, impacting tissue and whole body oxidant status. We hypothesized that 8 light-type horses would display improvements in skeletal muscle mitochondrial subpopulation function and decreases in circulating oxidant status markers during weight loss from an obese (7 to 8) to moderate (5) body condition score (BCS). Additionally, we hypothesized improvements in morphometric measurements, circulating metabolic markers, along with glucose tolerance and insulin sensitivity would also be seen. Change in BCS and percent weight loss were related to decreases in all morphometric measurements ($p<0.05$) except for abdominal circumference ($p<0.10$). Measurements of body composition also decreased with regard to both weight loss parameters ($p\leq0.001$). Circulating markers of lipid metabolism evaluated from non-fasting samples revealed no changes in non-esterified fatty acid (NEFA) or triacylglyceride concentration, yet ex vivo lipolysis media was decreased in response to weight loss ($p=0.07$) and BCS change ($p=0.01$). Insulin sensitivity calculated from baseline and post-weight loss intravenous glucose tolerance tests was improved after weight loss and corresponding BCS decline ($p\leq0.05$) while the disposition index, reflecting β–cell responsiveness, also increased due to weight loss ($p=0.09$). Plasma nitrate decreased in response to BCS reduction ($p=0.06$) and percent

weight loss ($p=0.06$), whereas erythrocyte total glutathione ($p=0.06$) concentration increased with decreasing BCS, signifying improvements in oxidant status. Mitochondrial electron transport chain complex I and IV displayed greater activity in SSM than IFM ($p\leq0.05$), while I, III, and IV in SSM had decreased activity due to BCS change and percent weight loss ($p\leq0.01$). Interactions between SSM and IFM complex IV activity and both weight loss markers ($p<0.05$) were displayed. Citrate synthase activity, indicating mitochondrial number, was greater in SSM than IFM ($p<0.0001$) but remained unchanged with weight loss parameters. Lipid peroxidation was decreased due to BCS change ($p=0.01$) and weight loss ($p=0.02$), with greater concentration in SSM, but a larger decrease in IFM ($p\leq0.05$). Alterations in complex activities and lipid peroxidation suggest IFM are more affected by weight loss, with large contributions from complex IV byproducts. Mitochondrial component flexibility may contribute individually to development and disease propagation along with athletic performance of the equine athlete. Lack of changes in circulating markers along with minimal changes in minimal model parameters suggest that while horses were obese, metabolic function was conserved. Further evaluation of metabolically healthy but obese horses may provide insight as to management and treatment of obesity associated maladies.

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Introduction

Obesity and its associated maladies plague both humans and horses in the United States today. Recent studies suggest nearly half the nation's horses may be overweight or obese, with 'easy-keeper' breeds at particularly high risk for unwanted weight gain (Geor et al., 2007; Henneke et al., 1983). A serious concern for horse owners and veterinarians, obesity is associated with equine metabolic syndrome and related conditions of insulin resistance, oxidative damage, chronic systemic inflammation, and laminitis (Geor et al., 2007). While knowledge of the effects of obesity on physiological pathways and metabolic parameters is growing, numerous areas still remain vague. Obesity is known to impact insulin sensitivity with confounding effects on glucose homeostasis, lipid metabolism, mitochondrial function and concomitant oxidative damage. Often, weight loss, as a result of caloric restriction and/or exercise, is touted to be the most beneficial means to decrease these effects of obesity; however, little is known about the impacts of obesity and the metabolic alterations occurring within an individual during stepwise decreases in body mass.

Obesity and the equine industry

Obesity affects a large population of the horses within the United States today. The prevalence of overweight and obese horses is much higher than initially estimated, with 41% of horses in the study found to be over conditioned or obese (Pleasant et al., 2008). By definition, obesity arises when energy intake exceeds energy expenditure, and this excess energy is primarily stored within the body as triglycerides or triacylglycerols

(Bray, 2004). Genetics greatly influence predispositions for this phenotypic trait as displayed by breed differences, but other management practices such as diet and physical activity, along with developmental stage and age also modulate this condition (Kopelman, 2000). For the feral equine, the ability to store energy can be a vital means for survival when food sources are scarce, yet for the domestic horse where dietary intake is often not a concern, extended periods of obesity or cyclical fluctuations in weight gain and loss may cause numerous metabolic maladies.

Although some horses may possess an overweight body condition for an extended period of time, others may gain and lose weight through the course of the year, in conjunction with increased or decreased work load (e.g. show season vs. winter), or seasonal alterations in diet such as the availability of lush pasture. Pasture can often meet or exceed the nutritional needs of many types of horses, despite seasonal variation between growing and dormant stages, along with diurnal alterations in nutrient status of grasses and legumes (NRC, 2007). Since starch content is contained within the chloroplast, starch saturation is easily achieved in warm season grasses due to chloroplast limitations, yet fructans are typically stored within vegetative structures, and can therefore be present in much higher concentrations as seen in cool season grasses (Bender and Smith, 1973; Ojima and Isawa, 1968).

Understanding and managing forage and concentrate intake for horses of any breed, nutritional status, or workload, is necessary to maintain adequate health as obesity and over-nutrition due to improper management poses numerous health risks. Managing the

obese horse can be challenging for many horse owners due to the multitude of problems that can arise regarding metabolic function. Weight loss is often the primary solution for the overweight horse; however, while much is known about the effects of obesity, little knowledge is available regarding the effects of weight loss after a bout of obesity. While the average horse owner is not concerned with the specifics of why obesity is harmful to the horse, knowledge of how obesity and subsequent weight loss affects metabolic parameters can educate those marketing nutraceuticals and pharmacological agents to the general public. In the obese horse, insulin insensitivity, hyperglycemia, and impaired lipid metabolism can give rise to dysfunction within the mitochondria and increased oxidative damage (Geor et al., 2007). In addition, further understanding of how the horse handles metabolic substrates on a cellular level can provide insight into fueling the entire body during times of excessive nutrients and caloric restriction, which can be important when managing both performance, and companion equines alike. Although a reduction in body condition to a moderate level is beneficial for the health of an animal or person, the malfunctions caused by obesity within glucose processing, lipid metabolism and mitochondrial function may have lasting effects. In order to gain a full appreciation for the implications of obesity on physiological function, a thorough understanding of the proper functions of these systems is warranted.

Normal metabolic function: nutrient intake and utilization in fed vs. fasting states

Glucose metabolism

Hormonal regulation is central to maintaining blood glucose concentrations at a relatively constant level. Balance between insulin, glucagon and other hormones is necessary to maintain metabolic homeostasis. Normal equine blood glucose ranges 40-70 mg/dL (Hoffman et al., 2003); inability to maintain this narrow range results in a multitude of detrimental changes to overall homeostasis within the body. For example, in horses prolonged elevation of glucose concentrations has a toxic effect on cells (Yki-Jarven, 1992), whereas low concentrations can produce seizures and disorientation (Valberg and Firshman, 2009). The effects of these hormones reach far beyond their role in glucose metabolism and many intermediates within these pathways feed into other catabolic and anabolic states. Prolonged periods of fed or fasting metabolism can cause obesity and insulin resistance, or starvation, respectively. Achievement of optimal body condition, performance and physiologic mechanisms is possible with proper management and understanding of the detailed hormone functions within these systems.

Circulating glucose can face multiple fates, including entry into the adipose tissue, skeletal muscle and liver. Glucose movement into the liver occurs through passive diffusion, where as uptake into adipose and skeletal muscle tissue is insulin dependent. Upon elevation of blood glucose concentration, glucose is transported into the pancreatic β-cell via facilitated diffusion though GLUT 2, an insulin independent transporter. Elevated blood glucose concentration is directly reflected in within the pancreatic β-cell and thus will elicit an appropriately large insulin response (Straub and Sharp, 2002).

Membrane depolarization occurs in response to glucose entry, leading to intracellular calcium release, which initiates a signaling cascade for the release of insulin-containing secretory vesicles via exocytosis (Straub and Sharp, 2002). Insulin is then able to reach skeletal muscle and adipose tissue via circulation and elicit glucose uptake to reduce blood glucose concentrations.

When insulin reaches the target tissue, binding of the hormone to the cell-surface insulin receptor induces autophosphorylation at a number of tyrosine residues on the receptor. Phosphorylation of specific residues allow for recognition by insulin receptor substrates (IRS), which are recruited to the receptor at the time of insulin binding and serve as docking sites. The insulin receptor then phosphorylates IRS at multiple tyrosine residues, allowing for recognition by the p85 regulatory subunit of phosphatidyl-inositol 3-kinase (PI3-kinase). Upon this activation, the p110 catalytic subunit of PI 3-kinase can then phosphorylate and convert phosphatidylinositol-2-phopshate (PIP2) to phosphatidylinositol-3-phosphate (PIP3) (Watson and Pessin, 2001). Protein kinase B is then activated by PIP3, which functions to recruit GLUT 4 containing vesicles to the plasma membrane. Facilitated diffusion of glucose into the cell can then occur, aiding in the decrease of blood glucose concentration. Translocation of GLUT 4 to the plasma membrane is primarily driven by insulin, and is thus considered an insulin dependent transporter (Goodyear and Kahn, 1998). Receptor sensitivity and effectiveness of the aforementioned signaling pathway is necessary to facilitate this response; any impairment can result in hyperglycemia and propagation of other associated maladies.

Lipid metabolism

Fatty acid (FA) metabolism is closely intertwined with glucose metabolism within the skeletal muscle and adipocyte and therefore must be considered when evaluating the effects of obesity on metabolic parameters. Fatty acid metabolism within the body involves a delicate balance between multiple steps including lipolysis and long chain fatty acid release from adipocytes, delivery of dietary or released fatty acids to muscle cells via transport across the plasma membrane, lipolysis of intramuscular triglyceride stores, activation of the fatty acid and transport into the mitochondria for oxidation (Holloway et al., 2009). In order to gain a big picture understanding of how fatty acid release from the adipose tissue and utilization of the substrate within the muscle is interrelated with obesity and weight loss consideration must be given to both adipose and muscle tissues, including individual mitochondrial subpopulations within the muscle. Further, to fully understand changes associated with excess lipid stores in the body, familiarity with lipid metabolism in the tissues and their contribution to energy production and intracellular signaling is necessary.

A typical ration of dietary forage and concentrate components would yield a variety of triacylglycerols, phospholipids and cholesterols, each contributing individually to the lipid profile of the horse's diet. De novo fatty acid synthesis occurs during times of energy excess, in the cytosol of liver and adipose tissue cells. In this biosynthetic pathway, condensation reactions are coupled with the hydrolysis of ATP causing the reaction to be driven to completion; ultimately leading to excess storage of fats in the adipose tissue.

In a fasted or other catabolic state such as exercise, chylomicron levels decline, and the adipose tissue becomes the major source of fatty acids for the body (Muoio and Newgard, 2006). A lower insulin-to-glucagon ratio, along with increased catecholamine levels due to a fasting/catabolic state, cause activation of hormone sensitive lipase (HSL). During lipolysis in the adipose tissue, HSL acts on diacylglycerols to release free fatty acids and glycerol into the circulation for use by other tissues. Concurrently, signaling within the body decreases glucose utilization while increasing fatty acid oxidation. Cytosolic citrate is used for synthesis of acetyl-CoA and malonyl-CoA, yet when acetyl-CoA carboxylase is phosphorylated and thus inactivated in the presence of AMP kinase, conversion of acetyl-CoA to malonyl-CoA ceases and concentrations decline. Low levels of malonyl-CoA combined with the effects of decreased citrate concentrations result in suppression of de novo lipogenesis and simulation of fatty acid oxidation through removal of malonyl-CoA inhibition of carnitine palmitoyltransferase-1 (CPT-1) activity. Elevated CPT-1 activity can ultimately lead to increased flux through fatty acid β-oxidation, as displayed in figure 1. Lastly, during times of increased fatty acid oxidation accumulation of acetyl-CoA, NADH and ATP allosterically inhibit pyruvate dehydrogenase. Overall, the actions of the mechanisms described during the fasting/catabolic state function to increase the rate of fat oxidation while decreasing glucose oxidation.

Mitochondrial metabolism

Mitochondria are double-membrane organelles specialized to convert energy dense macronutrients into ATP through oxidative phosphorylation, and also play a role in

various cellular signaling pathways including apoptosis, and cellular proliferation (Martinez, 2006). The production of ATP is achieved through oxidation of acetyl-CoA from two major sources: from the end products of glycolysis, which enter the mitochondrion as pyruvate and are converted to acetyl-CoA via pyruvate dehydrogenase, and fatty acid oxidation end products. The citric acid cycle then utilizes acetyl-CoA to form citrate from oxaloacetate by the action of citrate synthase, and is rate limited by the action of isocitrate dehydrogenase converting isocitrate into α-ketoglutarate. For each acetyl-CoA to enter the cycle, three $NADH^{+}$, a $FADH_2$ and an ATP are generated. The $NADH^{+}$ and $FADH_2$ are then able to carry electrons, or reducing equivalents, to the electron transport chain, where incremental releases of energy are applied to pump protons across the inner mitochondrial membrane. This is accomplished through the action of four major enzymes embedded within the inner mitochondrial membrane; NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), cytochrome bc1 complex (Complex III) and cytochrome c oxidase (Complex IV, figure 2).

Alterations in mitochondrial number are often needed to manage ever-changing energy substrate loads and metabolic demands placed on various tissues. Skeletal muscle mitochondrial biogenesis can be stimulated through the actions of peroxisome proliferator-activated receptor gamma (PPAR) coactivator1-alpha (PGC-1α) (Scarpulla, 2008). In its active form, PGC-1α interacts with nuclear receptor PPARs to increase gene transcription, along with the potential for PPARs to act in a feed forward fashion and increase PGC-1α further (Muoio and Newgard, 2006). It is through this interaction with PPAR that PGC-1α is able to increase mitochondrial biogenesis via increased

transcription of nuclear respiratory factors (NRFs), along with influencing oxidative phosphorylation gene regulation (Lin et al., 2002). Mitochondrial autophagy, or mitophagy, can also occur in response to cellular environmental factors or stressors. Utilization of this pathway can be activated in response to overwhelming oxidative damage, mitochondrial dysfunction or damage to critical proteins used for mitochondrial function (Lemasters, 2005). Mitochondrial number, whether in response to biogenesis or autophagy, can be indicative of overall aptitude of the tissue to respond to physiological stimuli along with providing a detailed image of disease states and energy production.

While mitochondria are needed in all cell types to provide ATP, skeletal muscle contains two sets of biochemically and spatially distinct mitochondria: subsarcolemmal mitochondria (SSM), which reside beneath the plasma membrane, and interfibrillar mitochondria (IFM) which are located between the myofibrils (Palmer et al., 1977). While they function to achieve the same general goal, differences have been found in their morphology, biochemical properties and response to physiological stimuli, including exercise and stress (Cogswell et al., 1993; Manneschi and Federico, 1995; Palmer et al., 1985). Subsarcolemmal mitochondria are located just beneath the sarcolemma and are larger, with a more lamellar shape, while IFM tend to be smaller, more compact and located between the contractile filaments (Koves et al., 2005). In response to exercise, SSM tend to display greater increases in volume, state III respiration and enzymatic activity than IFM (Bizeau et al., 1998; Kreiger, 1980), and during times of muscle disuse SSM show more pronounced decrements (Bizeau et al., 1998). Further, oxidative phosphorylation properties with respect to mitochondrial

subpopulation are different based on fiber type, and are individually affected in response to energy stress (Kim et al., 2002; Koves et al., 2005). Understanding the relationship between SSM and IFM and their contributions to skeletal muscle metabolism can be instrumental in understanding the alterations caused by increases or decreases in body condition, and therefore will be the focus of latter portions of this review.

Obesity-Related Changes in the Horse

Thermoregulation

While over-conditioned animals are suspected to be at higher risk for metabolic dysfunction, excess body fat may be advantageous to horses in cold climates. Body fat provides insulating properties along with providing an available energy source during times of low ambient temperatures. Previous studies have determined horses with a BCS of 7-9 require less supplemental feed during cold weather than horses with lower BCS (Cymbaluk and Christison, 1990). Conversely, this same property most likely induces heat stress in obese horses during hot/humid weather (Cymbaluk and Christison, 1990; Webb et al., 1989). Horses with a BCS of 7.5 or greater had a more difficult time dissipating heat in an ambient temperature of 31.5° C (humidity 43.8%) than horses with an average BCS of 5.2 (Webb et al., 1989). Further, these obese horses had a higher respiration rate following a light intensity exercise bout consisting of 30 min of mixed walk, trot and canter work. While thermoregulation is crucial to homeostatic maintenance of the horse, performance from both an athletic and reproductive standpoint is of great interest to the industry and recreational horse owners alike.

Athletic Performance

Currently within the equine industry, the impact of obesity on exercise performance has not been studied in depth. Preliminary research in this area showed a negative relationship between body weight and body fat percentage in performance measures of equine athletes (Thornton et al., 1987). In a study evaluating body composition of standardbred horses competing in one-mile races, larger amounts of body fat determined by rump fat ultrasound were negatively correlated to running performance, indicated by race times (Kearns et al., 2006b). Additionally, horses completing a 151km endurance race were found to have lower BCS and carried less body fat (26kg) than non finishers (45kg) (Lawrence et al., 1992).

Despite this valuable information, the previously mentioned studies were completed on horses with an average BCS of 4.5 and body fat percentage of 8.65% (Kearns et al., 2006b; Lawrence et al., 1992; Webb et al., 1989). It remains likely that horses with higher BCS would display further performance impairments compared to lean counterparts, especially if not enrolled in an exercise program.

Reproductive Performance

Body condition score to either an excessively high or low condition appears to impact estrous cycle length in broodmares, yet ovulation and fertility remain relatively unaffected with obesity. In mares with a BCS ranging from 7.5-8.5 continued to cycle during winter months, forgoing seasonal anestrus typically displayed by moderate and lean horses (Gentry et al., 2002). Higher circulating levels of leptin have been found in

obese mares (8.27 ± 1.02 ng/mL) compared to lean mares (~2 ng/mL), potentially contributing to the absence of an anestrous period in this cohort (McManus and Fitzgerald, 2003). This concept is further supported as when leptin levels declined, obese mares entered into an anestrous period. Cause of the decline during this study remains unknown, as it was not accompanied by a reduction of body fat percentage, but may have been affected by fluctuations in overall satiety (McManus and Fitzgerald, 2003). Conversely, other studies suggest excessive body fat in mares (BCS > 7) may prolong luteal phase length and interovulatory intervals when compared to thin mares (BCS 4) (Sessions et al., 2004). Elevated levels of leptin and insulin observed in the fatter mares, combined with the presence of insulin resistance were though to have caused the observed extensions (Sessions et al., 2004). Insulin resistance combined with other physiological indicators may be the influential factor between mares retaining normal reproductive cyclicity and the onset of dysfunction.

Guidelines for mares in gestation and lactation suggest a proper BCS before, during and after pregnancy should allow mares to be sufficiently fat so as to prevent loss in condition due to fetal development, but not so overweight as to induce metabolic stress (Quinn et al., 2006). Mares fed to either gain weight or maintain a BCS of 6.6-7.1 during gestation and lactation periods had higher successful pregnancy rates, decreased number of estrous cycles per conception compared to mares allowed to lose weight, with a BCS ranging from 6.1-3.7 (Henneke et al., 1984). Further, low levels of body fat (BCS <5) resulted in reduced pregnancy rates when compared to mares of increasing body condition (5-8), as mares with a higher BCS displayed decreased times to first estrous and first ovulation,

indicating improved reproductive efficiency (Henneke et al., 1984). Effects of obesity on reproductive capability in the stallion have not been fully evaluated. Obese stallions may have reduced sexual performance, potentially due more to the effects of obesity on athleticism and thermoregulatory properties over the impact of obesity on sperm production (Quinn et al., 2006). Influences of obesity on both mare and gelding metabolic function is often of a more pressing concern, as if the horse is able to remain in a healthy homeostatic state, then reproductive function will also be conserved.

Metabolic Impacts of Obesity

Obesity is known to cause alterations in transport and metabolism of various energy substrates, yet exact mechanisms for obesity-associated disturbances remain elusive. Obesity-induced insulin resistance can be linked to many pathologic distrubances within the skeletal muscle, ultimately resulting in disruptions in oxidative and storage pathways for glucose and lipid molecules. Insulin resistance by definition is a condition of low insulin sensitivity in which the ability of insulin to lower circulating glucose levels is impaired (Henry, 2003). Originally, insulin resistance was thought to arise from extended periods of hyperglycemia, and subsequent insulin release from the pancreatic beta cells. Elevated levels of both glucose and insulin over time cause receptor insensitivity to normal levels and as the cycle propagates the condition worsens. It is now known that multiple factors affect insulin resistance, including this pathway and others arising from disconcerted metabolic communication between tissues and cellular compartments.

Adipose tissue metabolism

Partitioning of lipids between adipose tissue and other peripheral tissues is integral in coordinating energy balance and signaling pathways regarding substrate utilization. As adipose tissue is designed to store triacylglycerides, enhanced lipolysis and free fatty acid flux from adipocytes exposes skeletal muscle and other tissues to a substantial fatty acid load, resulting in lipid accumulation in these tissues (Horowitz and Klein, 2000). Mircoarray experiments on obese leptin-deficient mice indicate genes encoding for transcription factors involved in lipogenesis, including sterol regulatory-element binding protein-1c (SREBP-1c) and PPAR-γ, are down-regulated in adipose tissue, yet are up-regulated in the liver (Nadler et al., 2000; Soukas et al., 2000). While PPAR-γ is not typically expressed at high levels in the liver (Nadler et al., 2000; Soukas et al., 2000), induction of transcription is caused by elevated circulating levels of triacylglycerides, suggesting relative expression is indicative of fuel repartitioning during times of obesity (Attie and Scherer, 2009). Moreover, adipose tissue expansion needed to accommodate excess nutrients places demands on vascularization and remodeling pathways. If these pathways lag behind the need for increased triacylglyceride storage, hypoxic conditions may emerge, leading to increased inflammation via macrophage recruitment (Attie and Scherer, 2009). Mitochondrial dysfunction, such as enzyme impairment or electron transport chain deficiency, in the adipocyte during times of hyperglycemia can also contribute to impairment of energy balance, as a decrease in respiratory capacity can reduce GLUT 4 translocation along with increasing reactive oxygen species production, further propagating local and systemic inflammation. (Lin et al., 2005; Shi et al., 2008).

Skeletal muscle glucose and lipid metabolism

The interplay of lipid metabolism with other energy substrates and signaling pathways is disconcerted in animals consuming diets in extreme excess of their current needs, or of those in an obese body condition. It has been well documented that obesity causes impaired insulin signaling and glucose tolerance (Hoffman et al., 2003; Kronfeld, 2005), manifested by decreased insulin-stimulated glucose transport and metabolism in skeletal muscle and adipocytes (Reaven, 1995) and increased levels of circulating free fatty acids (Kahn and Flier, 2000). In combination with decreased GLUT4 levels or translocation (Kahn and Flier, 2000) seen with an obese phenotype, dysfunction in the insulin signaling pathway is further propagated.

With the onset of obesity, triacylglycerides are not only stored in the adipose tissue, but begin to accumulate at unhealthy levels in the skeletal muscle, liver, kidney and heart (Muoio and Newgard, 2006) due to increases in circulating triacylglycerides and free fatty acids. Increased levels of fatty acid binding protein on the plasma membrane or fatty acid translocase/CD36 on the plasma membrane but not the outer mitochondrial membrane (Bonen et al., 2004) may play a critical role in increased fatty acid uptake, accumulation of intramuscular lipids and impaired insulin signaling (Consitt et al., 2009). Increases in intramuscular triglyceride stores, which, in addition to impairing GLUT4 translocation, may also propagate the development of insulin resistance (Montell et al., 2001). Further, long chain fatty acyl-CoAs are shunted away from CPT-1 and are instead are preferentially partitioned toward synthesis of signaling intermediates such as diacylglycerols and ceramide. Accumulation of these molecules combined with the

effects of excess intramuscular lipid stores are thought to activate serine-kinases (Holland et al., 2007; Yu et al., 2002), along with impacting protein kinase c and other downstream insulin signaling molecules (Schmitz-Peiffer, 2002), thereby preventing proper translocation of GLUT4 in an insulin-dependent manner. Without the necessary actions of this transporter, the insulin mechanism fails to reduce circulating glucose concentrations and, therefore, contributes to obesity-induced insulin resistance.

An imbalance between fatty acid uptake and fatty acid oxidation in obese individuals is often seen, contributing to the aforementioned impairment in glucose tolerance along with decrements in regulatory steps of fatty acid oxidation. Reduced activity of carnitine palmitoyltransferase-1 (CPT-1), β-hydroxy acyl-CoA dehydrogenase, citrate synthase and cytochrome c oxidase have been reported in samples of skeletal muscle from obese subjects (Simoneau et al., 1999), along with overall decreased mitochondrial content (Kim et al., 2000). Overexpression of CPT-1 in L6 cells caused an increase in fatty acid oxidation and protected cells against fatty acid induced insulin resistance (Muoio and Newgard, 2006), suggesting that increasing β-oxidation could help to correct maladies driven by this accumulation. Obese individuals have been reported to have an increased ratio of glycolytic to oxidative enzyme capacity, as determined by hexokinase to citrate synthase activity levels, yet not as elevated as the ratios seen in type 2 diabetic subjects (Simoneau and Kelley, 1997). Further, this ratio was negatively correlated with insulin sensitivity, providing increasing evidence that an alteration in metabolic preference may contribute to insulin resistance seen in an obese individual.

Mitochondrial function and oxidative status

While mitochondrial oxidative phosphorylation is a highly efficient process, a small amount of electrons can prematurely bind with oxygen to form free radicals. Further, under certain conditions such as obesity, excessive production of reactive oxygen species has been found and attributed to the mechanisms implicated in insulin resistance and metabolic syndrome (Martinez, 2006). A low degree pro-inflammatory state has also been associated with obesity, causing impairments in the oxidative stress and antioxidant mechanisms leading to activation of nuclear factor kappa-β in the presence of excessive reactive oxygen species (Martinez, 2006). Additionally, reactive oxygen species have been attributed to propagating insulin resistance and paracrine communication between adipocytes and macrophages, thereby mediating inflammatory changes by means of free fatty acids and tumor necrosis factor-α (Valerio et al., 2006).

Accumulation of intramuscular lipid may place additional strain on the mitochondria and create a larger disconnect between metabolic pathway fluidity. For example, a metabolic profiling study demonstrated that obesity-induced rates of β-oxidation overwhelmed the citric acid cycle, resulting in incomplete fatty acid degradation (Koves et al., 2008). Lack of cycle completion caused accumulation of mitochondrial-derived by-products, including acylcarnitines, which potentially can contribute to obesity induced insulin resistance. Moreover, electron transport chain activity in the vastus lateralis muscle, assessed from complex activities normalized to creatine kinase activity, in type II diabetic and obese human subjects was found to be diminished when compared to that of healthy lean counterparts, even when corrected for total mitochondrial content (Ritov et al.,

2005). Although the exact mechanism linking mitochondrial capacity and insulin resistance propagation is unknown, these findings suggest a link between insufficient coupling and/or reduced enzymatic activity in insulin resistant obese individuals.

As previously mentioned, skeletal muscle contains different populations of mitochondria which respond differently to physiological stimuli. In response to hyperglycemia and hyperlipidemia situations changes in individual size, population size, proteins involved in substrate utilization, electron transport chain (ETC) function, and reactive species production have been observed in rodents (Dabkowski et al., 2010; Dabkowski et al., 2008a). Typically, in an obese or type II diabetic model, the SSM tends to be more affected, while in a type I diabetic model, the IFM display greater dysfunction (Dabkowski et al., 2010; Williamson et al., 2009). The hyperglycemic environment caused in response to obesity and metabolic syndrome leads to enhanced reactive oxygen species (ROS) production, which can facilitate enhanced apoptosis along with causing oxidative damage to the cell and its contents (Williamson et al., 2009). Further, reduced activity of citrate synthase and CPT-1 in obese skeletal muscle has been used to infer reduced mitochondrial number (Kim et al., 2000), a concept confirmed by lower mitochondrial DNA in obese human individuals compared to lean counterparts (Ritov et al., 2005).

Despite contributions from other sources, the mitochondrion is regarded as the primary site of production of ROS (Kelley et al., 2002b). Several locations within the ETC are prone to formation of ROS, and due to the inclusion of iron-sulphur centers, which can

react with other ROS to produce the hydroxyl radical, can cause mitochondrial dysfunction (Fridovich, 1995). Dysfunctional mitochondria can result in further insult to the inner mitochondrial membrane, producing oxidative damage to lipids and proteins, which in turn limits adequate production of ATP. The effects of functional changes in mitochondria extend to fatty acid metabolism as well. In age-matched lean (BMI 23.3 ± 0.7) and obese (BMI 37.6 ± 2.2) humans, citrate synthase, a common marker of total muscle mitochondrial volume and capacity for fatty acid oxidation was decreased in obese individuals (Holloway et al., 2007). Due to the role of the mitochondria within metabolism and generation of ROS and implications of cellular apoptosis with corresponding dysfunction, subpopulations of mitochondria may be differentially affected by physiological parameters presented with obesity and subsequent weight loss. Inability for mitochondrial function to return to a normal functioning state could be detrimental for the animal, as prolonged periods of increased oxidative damage could lead to further disease and impairment in the future.

Differences in body condition

Effects of obese vs. lean body condition on metabolic function

As the skeletal muscle has the ability to transition easily between oxidative and glycolytic metabolism, this tissue adapts to a given set of metabolic cues, thus having the largest differences between obese and lean phenotypes. This malleable characteristic may allow skeletal muscle mitochondria to rebound after prolonged periods in an obese state. In a 16-week lifestyle modification program, obese non-diabetic volunteers were placed on a calorie-reduced diet and a moderate-intensity exercise program (Menkishova et al.,

2006). Significant improvements were noted with regard to insulin sensitivity, whole body fat oxidation and electron transport chain oxidative capacity. Despite this increase, mitochondrial number, as measured by citrate synthase activity and mitochondrial DNA, quantification was not altered.

It has been well documented that weight loss improves insulin sensitivity; however, little is known about the impact of weight loss on skeletal muscle mitochondria, potentially due to the recent development in knowledge on the effects of obesity on mitochondrial functions (Toledo and Goodpaster, 2013). Current research in this area appears to be confounding with respect to improving mitochondrial capacity. In a 1 yr weight loss study of obese women who lost an average of 55 kg, fatty acid oxidation was unchanged, along with mRNA content of CPT 1 and PGC-1α indicating mitochondrial biogenesis is not active during periods of weight loss (Berggren et al., 2008). Yet, in a study of obese women who had undergone moderate weight loss by caloric restriction and light exercise, succinate dehydrogenase activity was increased (Kern et al., 1999). Therefore, it is currently unclear if changes in mitochondrial function associated with weight loss are due primarily to alterations in metabolic function, or are manifested as a side effect of contractile activity during exercise.

Evaluation of markers of metabolism and oxidative damage through a weight loss period can provide a stepwise analysis of changes seen from an overall body perspective to a cellular level of function. Knowledge to be gained from this study has implications spanning multiple facets, as information on cellular changes are important to determine

how substrates are being handled on a chemical level, while implications of cellular products on body function is needed to elucidate the changes seen with regard to an increase and decrease in body condition. Within the equine industry, substrate utilization is needed for optimal performance along with maintenance, meaning the impact of data to be gained from this study has the ability to reach the performance industry and small farm practices alike. By developing a baseline understanding of the changes associated with weight loss on metabolic function, we can further evaluate the role of nutraceutical and pharmaceutical interventions designed to improve or adjust these parameters.

As of now, the majority of studies completed evaluating these parameters have been conducted either using two groups of animals: a lean and an obese group, or evaluating snapshot samples of pre- and post-weight loss. While this information is important in elucidating the damaging effects of obesity, little is known about how these parameters will change within an obese individual as body condition decreases over time.

FIGURES

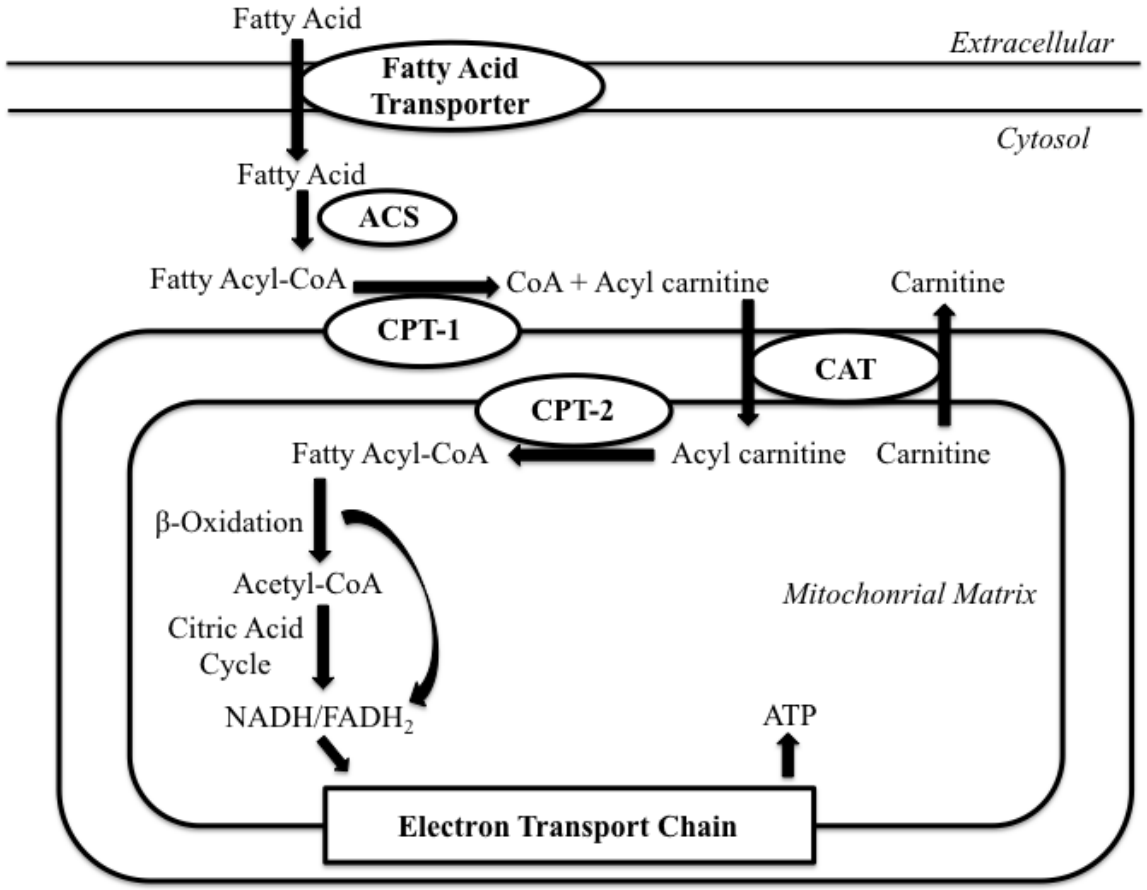

Figure 1: Entry of fatty acids into the mitochondria

ACS: Acyl-CoA synthetase, CPT-1: carnitine palmitoyltransferase-1, CPT-2: carnitine palmitoyltransferase-2, CAT: carnitine acyltransferase-1

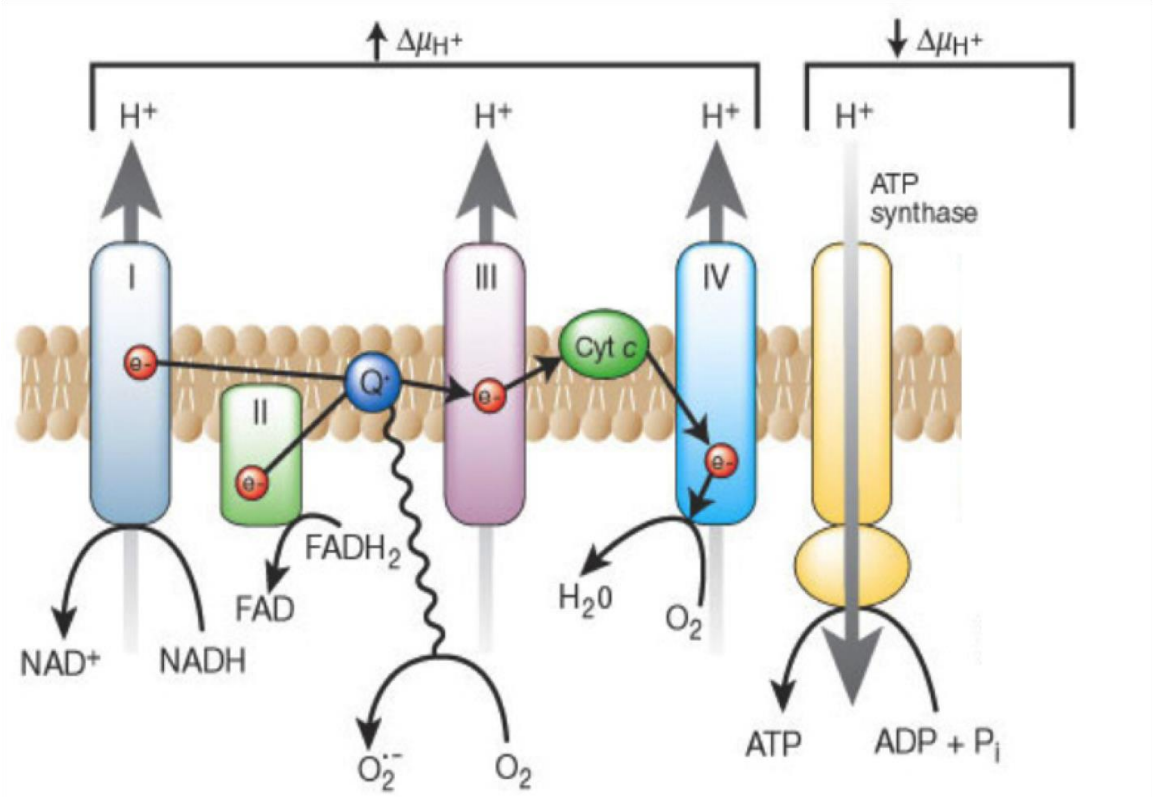

Figure 2: Electron Transport Chain Adapted from (Brownlee, 2001)

Q•: Ubiquinol, e-: electron, Cyt c: cytochrome c, ΔμH+: change in proton gradient

CHAPTER II: JOURNAL ARTICLE I

WEIGHT LOSS INFLUENCE ON NUTRITIONAL STATUS AND METABOLIC MARKERS IN A MATURE EQUINE MODEL

Abstract

Obesity is known to impact insulin sensitivity with confounding effects on glucose homeostasis, lipid metabolism, mitochondrial function and concomitant oxidative damage. Often, weight loss as a result of caloric restriction and/or exercise is touted as beneficial in decreasing these adverse effects of obesity; however, little is known regarding lasting effects of obesity on metabolic parameters after weight loss has occurred. We hypothesized that 8 light-type horses would display improvements in morphometric measurements, circulating metabolic markers, along with glucose tolerance and insulin sensitivity during weight loss from an obese (7 to 8) to moderate (5) body condition score (BCS). Effects of change in BCS or percent weight loss were evaluated using the mixed procedure of SAS with repeated measures. Change in BCS and percent weight loss were related to decreases in all morphometric measurements ($p<0.05$) except for abdominal circumference ($p<0.10$). Measurements of body composition also decreased with regard to both weight loss parameters ($p\leq0.001$). Circulating markers of lipid metabolism revealed no changes in non-esterified fatty acid (NEFA) or triacylglyceride concentration, yet ex-vivo lipolysis media was decreased in response to weight loss ($p=0.07$) and BCS change ($p=0.01$). Insulin sensitivity calculated from baseline and post-weight loss intravenous glucose tolerance tests was improved after weight loss and corresponding BCS decline ($p\leq0.05$) while the disposition index,

reflecting β–cell responsiveness, also increased due to weight loss (p=0.09). Lack of changes in circulating markers along with minimal changes in minimal model parameters suggest that while horses were obese, metabolic function was conserved. Further evaluation of metabolically healthy but obese horses may provide insight as to management and treatment of obesity associated maladies.

Key Words

Horse, weight loss, glucose, insulin, obesity

Introduction

Obesity among horses within the United States has reached an all time high, with nearly half of the nation's horses being considered either overweight or obese (Geor et al., 2007; Henneke et al., 1983). Obesity causes a multitude of metabolic issues in the horse, including impairment of glucose tolerance and insulin signaling, which compounded with compromised lipid metabolism and mitochondrial function can cause increased cellular damage and propagation of disease states. It has been well documented that obesity in the horse can cause impaired insulin signaling and glucose tolerance (Hoffman et al., 2003; Kronfeld, 2005), manifested by decreased insulin-stimulated glucose transport and metabolism in skeletal muscle and adipocytes (Kahn and Flier, 2000) and increased levels of circulating free fatty acids (Kahn and Flier, 2000). In combination with decreased GLUT4 transporter levels or translocation (Kahn and Flier, 2000) seen with an obese phenotype, dysfunction in the insulin signaling pathway is further propagated. As of now, the majority of studies completed evaluating these parameters have been

conducted using two groups of animals: a lean and an obese group. While the effects of obesity on physiologic pathways have been studied, many areas still remain vague, particularly with regard to the lasting effects of obesity on metabolic function and stepwise alterations in metabolic capacity during weight loss. Often, weight loss, as a result of caloric restriction and/or exercise, is suggested to be the most beneficial method to decrease these effects of obesity; however, little is known about potential lasting effects of obesity on metabolic parameters after weight loss has occurred. Therefore; the objective of this study was to characterize the effects of weight loss on whole body markers of glucose and lipid metabolism and insulin sensitivity. Additionally, we sought to determine the effects of weight loss on cellular markers and regulators of glucose and lipid metabolism, including both β-oxidation and lipid biosynthesis. We hypothesized that weight loss would improve markers of insulin sensitivity and glucose tolerance, while decreasing levels of circulating triglycerides and increasing levels of non-esterified fatty acids. Further, we suspected lipolysis within the adipose tissue and β-oxidation of the lipids in skeletal muscle would increase.

Materials and Methods

The Middle Tennessee State University Institutional Animal Care and Use Committee approved all methods and procedures used in this experiment. Horses were body condition scored by two trained individual reviewers and horses assigned a body condition score (BCS) of 7 or greater were admitted to the study. In addition, horses selected for this screening process were known to not have any predisposing metabolic conditions including Cushing's Disease or Metabolic Syndrome as defined for horses.

Eight, healthy, mature (5-19 yrs) light type mixed sex (n=6 mares, n=2 geldings) horses of either Quarter Horse, Standardbred or Tennessee Walking Horse breeds from the Middle Tennessee State University Horse Center herd were admitted based on these parameters for use in this study (table 1).

Sampling Periods and Diet

Prior to and during the study, all horses received the same type of mixed grass hay and commercial concentrate (Purina Strategy, Land O'Lakes Purina Mills, St. Louis, MO), with *ad libitum* access to water. Dietary analysis was measured on concentrate samples from 10 different bags and hay grab samples from at least 25 bales over 5 random dates during the study (Equi-analyticial, Ithaca, NY, table 2 and 3). Further, study horses were group housed in pastures with run-in shelters when not individually stalled over the entire study. Pastures did contain some plant life; however, there was not enough coverage to make a significant contribution to the diet and therefore was not included in digestable energy (DE) intake calculations or dietary analysis. Due to limiting labor factors, individual feed offerings and refusals were not measured daily, but estimated based on average flake weight and concentrate cup weight, taken from 10 total measurements.

The baseline sampling period (BSP) began on d0 and continued until d14, during which time horses were maintained on their current dietary intake, with a mean DE intake of 21.1 Mcal/d. Upon entry into the weight loss period (WLP, d15), horses received a 30% DE reduction by altering amount of concentrate and hay offered. Horses remained on this level of restriction until d43, at which time calculations were made for maintenance DE

requirements based on current body weight, as described by the equation (NRC, 2007) DE (Mcal/d) = 1.4 + (0.03 x BW) to achieve steady weight loss . In order to facilitate further weight loss, a 30% reduction from the maintenance requirement was calculated and implemented throughout the remainder of the study (mean DE intake 14.01). At no point during the study did horses consume less than 1.5% BW in forage per day to ensure proper hindgut health.

During both the BSP and the WLP, all horses were subjected to exercise not exceeding the parameters of light workload as defined by the NRC. Horses were exercised on average between 1-3 hr/wk, with an approximate gait breakdown of 40% walk, 50% trot and 10% canter. Horses not broke to ride were lunged or worked in a round pen.

Sample Acquisition & Analysis

Body Condition Score, Cresty Neck Score and Morphometric Measurements

Two individuals assigned a body condition score (BCS) based on a 1-9 scale (Henneke et al., 1983) and a cresty neck score (CNS) on a 0-5 scale (Carter et al., 2009). Scores were assigned on a whole or half score basis and the average was used for statistical analysis. Morphometric measurements consisted of body weight, wither height, body length, neck length (NL), neck crest height, neck circumference at 0.25, 0.5, and 0.75 of NL, girth circumference, and abdominal circumference, in accordance with the measurements made by Carter and colleagues (Carter et al., 2009). Measurements, BCS and CNS were made on d 0, 14, 28, 42, 70, and 98. Body length was measured from the intermediate tubercale of the humerous (point of the shoulder) to the ischiatic tuberosity (point of the

buttock). All neck measurements were made with the neck in a relaxed position, at an approximate 45° angle. Neck length was measured from the poll to the highest point of the wither. Neck crest height was measured at 0.5 neck length, from the differentiation of musculature and tissue above the nuchal ligament (assessed by palpation and visual identification) to the dorsal midline of the neck. Neck circumferences were measured at 0.25, 0.5 and 0.75 of neck length. Girth circumference was measured immediately behind the slope of the wither, caudal to the elbow (olecranon tuber). Abdominal circumference was taken at two-thirds the distance from the intermediate tubercale of the humerous (point of the shoulder) to the point of the hip (tuber coxae). Horses were clipped in the areas where measurements were made initially to ensure later measurements were taken at the same location.

Body Composition Measurements

Rump fat thickness (RFT) was measured using B-mode ultrasound (Sonovet 2000, 5mgHz) (Kearns et al., 2006a; Kearns et al., 2006b) on d 0, 14, 28, 42, 70, and 98. The site for measurement was determined by measuring half the distance from the point of the hip (tuber coxae) to the point of the buttock (ischiatic tuberosity); and half of the distance from this point to the spine. Scans were made on alternating sides so as not to be affected from healing biopsy sites. Body fat percentage was estimated using the following equation (Kane et al., 1987); Percent Fat = 2.47 + 5.47 (rump fat in cm). Fat mass was calculated by multiplying percent fat and total body mass. Fat free mass was determined by the difference between total body mass and fat mass.

Circulating Metabolic Biomarkers

Non-fasting blood samples (~35 mL) were collected via jugular venipuncture on d 0, 14, 21, 28, 35, 42, 56, 70, and 98, placed into serum and sodium heparin vacutainers (Vacutainer, Franklin Lakes, NJ). Sodium heparin tubes were immediately placed on ice, while serum samples were allowed to clot at room temperature for at least 20 min before being centrifuged at 3000 x g for 15 min. Plasma and serum aliquots were removed and stored at -20° C until analysis. Serum non-esterified fatty acid (NEFA) and plasma triacylglyceride (TG) concentrations were measured in duplicate using a commercially available kit (HR-NEFA and L-Type TG Wako Diagnostics, Richmond, VA) as instructed by the kit insert. The coefficient of variation accepted between duplicates was 5% and 10% for NEFA and TG respectively (O'Connor et al., 2007; O'Connor et al., 2004). Glucose (Autokit glucose, Wako Diagnostics, Richmond, VA) and insulin (Coat-A-Count Insulin, Siemens, Los Angeles, CA) concentrations were also determined using commercially available kits, where the accepted coefficient of variation between replicates was 5% and 10%, respectively.

Frequently Sampled Intravenous Glucose Tolerance Tests (FSIGT)

All horses were subjected to a FSIGT on d 12 or 13 (end of BSP, FSIGT 1) and again at the end of the WLP (FSIGT 2). As all horses did not exit the study on the same day, FSGIT 2 was administered within 3 d of final morphometric measurements, biopsies, and blood sampling. Concentrate feeding was withheld for at least 12 h prior to the test but horses had access to grass hay the morning of and during the FSIGT. The protocol for

this test was determined from previously published methods, described in detail below (Hoffman et al., 2003).

Horses were weighed the morning of the test using an electronic scale and catheterized (Abbocath, 14g, 5.5 in, Abbott Laboratories, Abbott Park, IL) using aseptic technique and lidocaine anesthesia between 0800 and 0930. Horses were grouped randomly to allow for test staggering if multiple horses were to be tested on the same day. A glucose bolus, given at 0.3g/kg BW (50% dextrose solution, Phoenix Pharmaceutical, Inc, St. Joseph, MO), was infused via catheter within a 2 min period. Timing for all other samples began after the infusion finished, where the 1 min sample signifies the collection of a sample 1 min post glucose infusion, et cetera. Twenty min post-glucose infusion, an exogenous 30 mIU/kg BW insulin bolus (Humulin R, Willow Birch Pharmaceutical, Inc, Taylor, MS) was administered through the catheter. After each dose, the catheter, extension set and stopcock were thoroughly flushed with heparinized saline.

The test was conducted over ~4 h, during which blood samples (~25 mL) were collected at the following time points: -30 (baseline), 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 min post glucose administration. An additional 240 min time point was sampled during FSIGT 1 to allow adequate time for blood glucose to return to baseline for a more appropriate representation of the data when completing Minimal Model analysis.

Samples were collected into sodium heparin or serum tubes (Vacutainer, Franklin Lakes, NJ). Sodium heparin tubes were placed immediately on ice while serum tubes were allowed to clot at room temperature for at least 20 min before being placed on ice. All tubes were centrifuged at 3000 x g for 15 min. Aliquots were removed and stored at -20° C until analysis. To determine insulin concentration, serum aliquots were analyzed in duplicate using a commercially available radioimmunoassay (Coat-A-Count Insulin, Siemens, Los Angeles, CA) as directed by the package insert. After the decanting step, tubes were placed in a gamma counter (Perkin Elmer, Wallac 1470 Wizard Auto Gamma-counter, Waltham, MA), where each replicate was counted for 1 min. Plasma aliquots were measured in duplicate using a commercially available kit (Autokit Glucose, Wako Diagnostics, Richmond, VA) adapted to a 96 well plate (Nielsen et al., 2010). In brief, 9 μL samples, standards, and controls were each loaded into the plate, followed by 300 μL of buffer solution. Plates were then incubated for 2 min, and read on a UV spectrophotometer (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA). Each of these kits have been previously validated for equine samples (Hoffman et al., 2003). The accepted coefficient of variation between replicates was 5% and 10% for glucose and insulin, respectively (Nielsen et al., 2010). Area under the curve (AUC) for glucose and insulin was calculated using the trapezoidal method, with individual data points from each FSIGT (Nielsen et al., 2010). The Minimal Model Software Program (MinMod Millenium, Version 6.2) (Boston et al., 2003) was used to calculate glucose effectiveness (Sg), insulin sensitivity (Si), acute insulin response to glucose (AIRg) and disposition index (DI). Baseline glucose (GB) and insulin (IB) are calculated using initial baseline glucose and insulin values in addition to values of each curve after returning to baseline

at the end of the test. The highest concentration of glucose after intravenous injection is noted as G0. Any animal that did not fit the model with an R^2>0.9 was removed from the model prior to statistical analysis (FSIGT 1; n=1, FSIGT 2; n=0).

Neck Fat Biopsies

Neck fat biopsies were collected on d 0, 14, 28, 42, 70, and 98. Prior to biopsy collection, horses had biopsy sites clipped and scrubbed with chlorhexadine solution followed by an isopropyl alcohol rinse. Horses were then sedated with xylazine (0.5mg/kg BW) and were administered lidocaine anesthesia at the neck (2.5cc) site. Alternating sides were used for consecutive biopsies so as to allow time for sites to fully heal before further sampling occurred.

Neck fat biopsies were obtained at approximately 0.5 neck length (measured as described above), centered between the differentiation of musculature and tissue above the nuchal ligament using aseptic techniques (Liburt et al., 2012). A small incision, approximately 2 cm, was made through the skin and connective tissue to allow access to the neck fat pad. An adequate sample was held with a hemostat and dissected out using a scalpel blade. Incisions were closed with staples, left to heal for 10 d, when staples were removed. Samples were taken on alternating sides per biopsy to allow sites to fully heal. Post-collection, adipose tissue samples were weighed and allocated for an ex vivo lipolysis assay conducted immediately.

Lipolysis Assay

166 The lipolysis assay was conducted as described previously (Ippagunta et al., 2011) with
167 slight modifications. A minimum of two 100 mg pieces of adipose tissue were incubated
168 in Ringers buffer, with added BSA (fatty acid free, Sigma-Aldrich, St. Louis, MO) for 3 h
169 at 37° C in a shaking water bath. Media sampled were collected and stored at -20° C until
170 analysis for non-esterified fatty acids (NEFA, NEFA-HR kit, Wako, Richmond, VA) and
171 glycerol (free glycerol reagent, Sigma-Aldrich, Inc., St. Louis, MO) via colorimetric
172 analysis following the kit instructions. Accepted coefficient of variation between
173 duplicates was 5% and 10% for NEFA and glycerol, respectively. Concentrations of
174 NEFA and glycerol were adjusted for tissue weight and are expressed as mean μmol
175 released per g of tissue.
176
177 ***Statistical Analysis***
178 Data are expressed as mean ± SEM. Normality of all data were assessed prior to
179 statistical analysis (SAS Institue, V9.3, Cary, NC). Any variable found to be non-
180 normally distributed by evaluation of kurtosis and skewness values, along with
181 significance in tests for location (students t and sign tests) and normality (Shapiro-Wilk
182 and Klomogorov-Smirinov tests) where $p \leq 0.05$, were $\log_{10}$ transformed. The effect of
183 BCS change and percent weight loss was tested for all data using proc MIXED procedure
184 of SAS with repeated measures. Gender, horse, age and date were included in the class
185 statement during the repeated measures analysis. The model with the best fit according to
186 Akaike's Information Criterion used a compound symmetry structure. A Pearsons
187 Correlation was run using the proc CORR procedure of SAS, with Bonferroni correction
188 to prevent the incidence of type I errors when performing multiple comparisons. For all

data except correlations, $p<0.05$ was considered significant and trends were noted at $p<0.10$. Due to the bonferroni correction, $p<0.0032$ was considered significant and trends noted when $p<0.0047$ for all correlations.

Results

Body, Morphometric and Composition Measurements

A summary of individual horse characteristics including age, sex, breed, wither height, body length and neck length can be found in table 4. Horses on the study transitioned from a mean BCS of 7.81 ± 0.13 to a moderate body condition, 5.0 ± 0.0 during the course of the weight loss period. Change in body condition score and weight loss were related to decreases in girth circumference ($p<0.04$), abdominal circumference ($p<0.08$), cresty neck score ($p<0.0001$), neck crest height ($p<0.0001$), and all neck circumference measurements ($p<0.01$, table 5). Mean body weight decreased during the weight loss period from 548.41 ± 28.74 kg to 501.36 ± 27.81 kg, corresponding to decreasing BCS during this time. Similarly, rump fat thickness, percent body fat, fat mass and fat free mass decreased in relationship to both weight loss parameters ($p\leq0.001$) and are summarized by day in table 6.

Circulating Metabolic Markers

Evaluation of serum NEFA (figure 1A) and plasma TG (figure 1B) as markers of alterations in lipid metabolism revealed no differences due to change in BCS or percent weight loss. As markers of carbohydrate metabolism, glucose concentration (figure 1C)

displayed a significant increase when evaluated in relationship to change in BCS ($p=0.01$) but not with regard to percent weight loss. Conversely, insulin concentration (figure 1D) did not differ with change in BCS, but displayed a trend when evaluated with percent weight loss ($p=0.06$).

Lipolysis

Media collected from the lipolysis assay displayed a trending decrease in NEFA release due to weight loss ($p=0.07$) and BCS change ($p=0.01$, figure 2A). Free glycerol concentration from the same samples did not differ due to either weight loss factor ($p>0.14$, figure 2B).

FSIGT and Minimal Model Parameters

Minimal model parameters calculated from FSIGT glucose and insulin concentrations displayed variable results with regard to statistical model components (table 7). Glucose effectiveness (Sg) or AIRg were not different due to weight loss parameters. Despite this result, Si displayed an increase due to BCS change and percent weight loss ($p<0.04$). In contrast, DI displayed no relationship to change in BCS, yet a trend was noted with regard to percent weight loss during the study ($p=0.09$). Baseline glucose (GB), as calculated from beginning and end of the FSIGT decreased, with a trend noted for BCS change ($p=0.06$) and a significant difference in response to weight loss percentage ($p=0.002$). Baseline insulin (IB) and G0, the highest concentration of glucose post-intravenous injection, were not different due to either model parameter for weight loss ($p>0.21$).

Area under the curve for glucose and insulin during the FSIGT was calculated utilizing the trapezoidal method, and analyzed in accordance with other variables. Glucose AUC (figure 3A) decreased in response to percent weight loss ($p=0.0004$) and change in BCS ($p=0.05$). A similar effect was seen for insulin AUC (figure 3B), where when analyzed with regard to BCS change, a significant difference was detected ($p=0.02$); however, when analyzed for effect of percent weight loss, no difference was found.

Correlations

Correlations between collected data were analyzed in two separate sets: (1) analyzing all morphometric measurements, body composition data, circulating metabolic markers and lipolysis media concentrations of NEFA and FG, and (2) analyzing only data collected on days 14 and 96 including all minimal model parameters in accordance with the dates FSIGT were completed. A summary of correlations described in set 1 can be found in table 7. Weight was found to be correlated to girth circumference ($r=0.84$), abdominal circumference ($r=0.62$), neck circumference at 0.25 ($r=0.79$), 0.5 ($r=0.69$), and 0.75 NL ($r=0.76$), and fat free mass ($r=0.99$), where $p<0.0001$ for all correlations. A trend was also noted for the correlation between fat mass and weight ($r=0.42$, $p=0.003$). As body condition score decreases, a trending correlation with decreasing lipolysis NEFA concentration ($r=0.43$, $p=0.004$) was displayed.

Many of the same correlations were noted when comparing the aforementioned variables with minimal model parameters; however; due to a more stringent Bonferronis correction

as more variables were included in the correlation, only a few significant relationships were determined. Body condition score ($r=0.75$, $p=0.001$) and cresty neck score ($r=0.73$, $p=0.001$) were found to have positive correlations with AUC glucose. Area under the insulin FSIGT curve displayed positive correlations with fat mass ($r=0.71$, $p=0.0019$) and Gb ($r=0.80$, $p=0.0002$, data not shown).

Discussion

Body composition is an important factor to consider when evaluating metabolic function, specifically when related to glucose metabolism (Pedersen et al., 2003; Reaven, 1995). Horses included in the current study displayed decreases in all morphometric and body composition measurements during weight loss. As the Henneke body condition scoring system is subjective and can vary slightly due to each independent assessor (Henneke et al., 1983), morphometric measurements were also included to provide a more quantitative, comprehensive representation of regional adiposity differences during weight loss (Carter et al., 2009). Baseline mean cresty neck scores were similar to those found in insulin resistant ponies (Tinworth et al., 2012); yet overall BCS of the ponies was 6.0 ± 0.9, signifying a greater amount of regional adiposity than our horses which were at a greater BCS. In accordance with decreasing body condition score, all morphometric measurements including girth circumference, neck crest height, and 0.25, 0.5 and 0.75 neck circumference decreased.

Rump fat thickness is a measured parameter directly reflecting fat mass in the body (Westervelt et al., 1976). The concurrent decrease in rump fat with body weight suggests

horses were loosing fat mass during the course of the study. Calculations of fat mass and fat free mass also displayed patterns reflecting this concept, whereas body weight decreased, fat mass decreased from 46.37 ± 4.39 to 29.11 ± 3.10 kg and fat free mass declined 502.04 ± 27.14 to 472.25 ± 26.54 kg. The decrease in fat free mass suggests utilization of lean body mass sources for energy homeostasis consistent with calorie restriction. The light exercise program was not rigorous enough to increase muscle mass and as horses were accustomed to this level of activity no muscular adaptation to workload was expected.

Compared to other morphometric measurements, abdominal circumference displayed the smallest decrease over time, which may play a role in the lack of fluctuations seen in metabolic markers and minimal model parameters. In humans, intraabdominal fat deposits appear to have a direct role in disrupting glucose and lipid homeostasis (Leibel et al., 1989). As intraabdominal deposits drain directly into the portal vein, thereby resulting in high concentrations of hepatic free fatty acids which may interfere with hepatic catabolism of insulin along with overproduction of very low density lipoproteins (Leibel et al., 1989; Smith et al., 1985). The resultant increase in circulating insulin concentration may propagate insulin insensitivity over time. It is possible that during the process of fat deposition in the horse, abdominal fat is deposited last and also metabolized later during the weight loss process. In a study evaluating fat deposition in response to different dietary sources, the greatest amount of fat lay down occurred in the neck, with the smallest amount over the withers and loin (Suagee et al., 2008). Comparing this concept to neck crest fat, where a higher CNS is often linked to metabolic

syndrome predisposition (Carter et al., 2009; Treiber et al., 2006), it is plausible that due to the moderate CNS scores (2-3) seen during this study, the horses had not deposited enough fat to cause alterations in many of the metabolic parameters measured.

Additionally, specific fat deposits have varying metabolic functions and therefore may contribute differently to energy homeostasis during weight loss (Liburt et al., 2012). Within the horse, it is possible that the role of neck fat relating to homeostatic function gives an accurate yet localized overview of adipose tissue function in this model. As the neck fat crest is easy to access for sampling purposes, many groups have analyzed the metabolic function of this fat deposit (Liburt et al., 2012). Despite its value due to ease of accessibility, analysis of other fat deposits may be instrumental to fully elucidating the contributions of each deposit and changes associated with weight loss within adipose tissue. We found a decrease in NEFA release due to both weight loss parameters but no change in free glycerol during our measurement of lipolysis. These differences may be due to the 3 to 1 ratio of fatty acids to the glycerol backbone in TG (Ippagunta et al., 2011). Combined with the lack of differences in serum NEFA and plasma TG with either weight loss parameter, it is possible that while horses were overweight, the excess condition did not cause unhealthy lipid metabolism.

Additionally, a potential explanation for the lack of differences in circulating NEFA and TG values is that horses were not fasted at the time of sampling. Samples obtained from non-fasting horses displayed lower levels of NEFA (O'Connor et al., 2007) due to alterations between fed and fasting state metabolism. While most of the horses on the

study were receiving mainly grass hay and a very small quantity of concentrate, the possibility remains that if horses had been sampled prior to morning feed times different results may have been displayed. This would explain the elevated glucose concentrations that would otherwise suggest horses were hyperglycemic both during baseline and post-weight loss. Despite the significant difference in glucose concentration due to change in BCS and the trend noted for insulin concentration in relationship to percent weight loss, it is difficult to determine if this was a true weight loss response or a result of time of sampling post feeding. A more accurate representation of glucose and insulin concentrations due to weight loss is observed during the FSIGT, and will be discussed further.

As a better representation of fasting glucose concentration, Gb calculated from beginning and end of each FSIGT glucose concentrations, displayed significant decreases due to both weight loss and BCS change. This suggests that while initial alterations to glucose metabolism may have been occurring, they had not advanced to implicating other pathways measured. Upon evaluation of minimal model parameters along with glucose and insulin curve responses it is apparent that large individual variability is observed. While Si displayed an improvement, the lack of change in Sg, or AIRg suggest that while horses were obese, carbohydrate metabolic function was not entirely compromised. Increases in Si, the capacity for insulin to promote glucose disposal (Hoffman et al., 2003), can be contributed to weight loss, as in previous studies low intensity exercise alone did not produce improvements in Si (Carter et al., 2010). Interestingly, AIRg values observed during baseline when horses were obese are similar to those of moderately

obese horses (BCS 6-6.9) observed by Hoffman and colleagues (Hoffman et al., 2003). After weight loss, AIRg values were consistent with those reported in literature for horses with a BCS range of 5-5.9 (Hoffman et al., 2003). The trend observed between DI and percent weight loss may represent initial changes in endogenous insulin release, which may have been amplified if a larger sample size had been used. Previously reported literature demonstrates DI between horses of non-obese (BCS 5.0-5.9) and obese (BCS 7-9) condition did not differ, suggesting adequate ability in obese animals for AIRg to compensate for limited Si (Hoffman et al., 2003); a phenomenon which also may be present as horses transition through decreasing body condition scores.

Some variability between FSIGT 1 and 2, along with intraassay variation may additionally contribute to the lack of difference between FSIGT dates. In a previous study comparing multiple methods for determining insulin sensitivity and glucose tolerance, repeated tests displayed Si, Sg and AIRg mean coefficient of variation of 24%, 26% and 12%, respectively (Pratt et al., 2005). While utilization of the FSIGT and minimal model analysis is still an accurate measurement of glucose and insulin dynamics, it is important to consider the role of variability when interpreting results. In order to represent individual horse differences in FSIGT responses during baseline (date 1) and after weight loss (date 2) figure 4 depicts glucose (A) and insulin (B) curves. Horse numbers correspond to those in table 1. Horse variability greatly influenced the results of this study. Although no statistical differences were noted for many of the minimal model parameters, the physiological implications of individual horse responses to the FSIGT hold value in understanding how the body responds to decreases in body fat and weight.

When evaluated as a whole, significant differences in area under the curve for both glucose and insulin concentrations suggest that horses were compromised in some aspect of carbohydrate metabolism, but not to the extent to produce statistical differences in minimal model compartment parameters.

When evaluating the overall implications of this study, it remains plausible that while horses were considered obese at the start of the study, from a metabolic perspective they were not unhealthy. The term "metabolically healthy obese" has been coined in human health to describe obese individuals exhibiting relatively normal metabolic functions despite being as overweight as those presenting with metabolic dysfunction and nutritional diseases (Bonora et al., 1991; Sims, 2001; Wildman et al., 2008). It is hypothesized that the incidence of this phenotype is relatively prevalent, with up to 30% of the adult obese population possessing reduced central adiposity, lower adipocyte stress and decreased inflammation (Denis and Obin, 2013; Kloting et al., 2010). While insulin action is preserved in these subjects despite increasing body mass index (Bluher, 2012; Bluher, 2010; Kloting et al., 2010), it is not yet understood whether these individuals are genetically predisposed to maintain insulin sensitivity or whether the phenotype is inherently unstable and can evolve rapidly to an insulin resistant state (Kim and Reaven, 2008). Despite horses included in this study beginning at an obese BCS, relatively moderate CNS scores may signify a similar phenomenon within the selected population. Horses with a greater adipose deposition may produce a different outcome, as horses may transition into an unhealthy phenotype during this time. By selecting for horses without history of laminitic issues or known insulin resistance, we may have chosen a subset of

horses with resistance to developing metabolic dysfunctions. Moreover, this may suggest that while obesity is not a desired phenotype for the horse, that animals enrolled in an exercise program may be additionally protected from the onset of insulin resistance and other obesity-related metabolic maladies.

Looking forward, increasing the sample size for a study evaluating weight loss markers may be necessary to both eliminate confounding effects of horse-to-horse variability along with obtaining samples both during weight loss and weight gain. As many of the studies involving FSGIT with minimal model analysis and other metabolic studies have used between 6-10 horses, we suspected that this sample number would be adequate to elucidate changes due to weight loss as well (Hoffman et al., 2003; Liburt et al., 2012; Pratt et al., 2005; Tinworth et al., 2012). Also, collected data from this study could be utilized for a power analysis to determine sample number for future work in this area.

In conclusion, horses may exhibit a metabolically preserved obesity phenotype that is relatively protected from alterations in glucose and lipid metabolism. Deposition of fat more equally throughout the body as opposed to large deposits in the neck crest or intraabdominal regions may contribute to this phenomenon. Additionally modifications to insulin sensitivity and thus glucose and insulin concentrations in response to weight loss during an FSIGT may signify beginning metabolic changes that if horses were allowed to deposit more adipose tissue may cause further impairment over time. Future studies are needed to examine effects of the length of time in an obese state as well as stepwise

418 changes in weight gain in order to fully elucidate mechanisms behind obesity induced
419 metabolic dysfunction.
420
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427 assistance in the completion of this project.
428
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Tables and Figures

Table 1: Summary of individual characteristics of horses enrolled in study

Horse	Age (yr)	Sex[1]	Breed	Wither Height (cm)[2]	Body Length (cm)[3]	Neck Length (cm)[4]
1	10	M	Quarter Horse	142	158	71
2	6	M	Tennessee Walker	148	137	71
3	13	M	Standardbred	160	156	101
4	19	G	Quarter Horse	149	150	86
5	5	M	Quarter Horse	147	152	81
6	10	G	Quarter Horse	170	165	97
7	19	M	Quarter Horse	151	159	95
8	15	M	Quarter Horse	157	165	104
			Mean ± SEM	153 ± 3	155 ± 3	88 ± 4

[1] For sex categorization M represents mare and G represents gelding.

[2] Measured from the ground to the highest point of the wither

3 Measured from the point of the shoulder to the point of the rump

4 Measured from the poll to the point of union between neck musculature and the wither

439 Table 2: Nutrient analysis of grass hay and concentrate, as fed

Nutrient, as fed	Grass Hay	Concentrate
Dry Matter (%)	95.8	93.4
Digestable Energy (DE, Mcal/kg)	1.85	2.84
Crude Protein (g/kg)	51.5	150.1
Acid Detergent Fiber (ADF, g/kg)	371.8	166
Neutral Detergent Fiber (NDF, g/kg)	625.8	330.8
Water Soluble Carbohydrates (WSC, g/kg)	56.0	89.4
Ethanol Soluble Carbohydrates (ESC, g/kg)	15.5	51.7
Starch (g/kg)	14.3	131.4
Non-fiber Carbohydrates (NFC, g/kg)	156.8	350.9
Crude Fat (g/kg)	28.6	59.3
Calcium (g/kg)	6.03	12.91
Phosphorus (g/kg)	0.83	8.15
Sodium (g/kg)	0.218	2.369

440

441 Table 3: Percent composition of diet on a dry matter basis

Nutrient, % dry matter	Grass Hay	Concentrate
Crude Protein, %	5.4	16.1
Acid Detergent Fiber, %	38.8	17.8
Neutral Detergent Fiber, %	65.4	35.4
Water Soluble Carbohydrates, %	5.9	9.6
Ethanol Soluble Carbohydrates, %	1.6	5.5
Starch, %	1.5	14.1
Non-fiber Carbohydrates, %	16.4	37.6
Crude Fat, %	3	6.3
Calcium, %	0.63	1.38
Phosphorus, %	0.09	0.87
Sodium, %	0.023	0.254

442

443

444 Table 4: Morphometric measurements during weight loss

Variable		Day						P-Value: Change in BCS	P-Value: Percent Weight Loss
		0	14	28	42	70	98		
Body Condition Score[1]	Mean	7.81	7.75	7.44	7.38	6.13	5.00	.	.
	SEM	0.13	0.13	0.18	0.23	0.25	0.00		
Girth Circumference[2,3]	Mean	194	195	194	193	193	189	0.04	0.01
	SEM	4	4	4	4	5	3		
Abdominal Circumference[2,4]	Mean	215	212	208	206	202	199	0.08	0.06
	SEM	4	4	4	10	7	5		
Cresty Neck Score[5]	Mean	2.69	2.63	2.64	2.56	2.19	2.07	<0.0001	<0.0001
	SEM	0.09	0.08	0.13	0.15	0.09	0.07		
Neck Crest Height[2.6]	Mean	12	12	10	9	9	7	<0.0001	<0.0001
	SEM	1	1	1	1	1	1		
Neck Circ. at 0.25 NL[2,7]	Mean	79	80	78	77	77	74	0.002	0.01
	SEM	2	1	2	2	1	2		
Neck Circ. at 0.5 NL[2,7]	Mean	101	99	94	94	96	91	0.003	0.002
	SEM	3	3	2	2	3	1		
Neck Circ. at 0.75 NL[2,7]	Mean	118	117	112	110	114	108	0.003	<0.0001
	SEM	3	3	3	3	4	3		

445

446 [1] Assessment based on a 1-9 scale (Henneke et al., 1983)

447 [2] All measurements made in cm

448 [3] Measured immediately behind slope of wither, caudal to elbow

449 [4] Measured at two thirds distance from point of shoulder to point of hip

450 [5] Assessment based on 1-5 scale (Carter et al., 2009)

[6] Measured at 0.5 neck length (measured from poll to highest point of the wither), from the differentiation of musculature and tissue above the nuchal ligament (discerned from visual and palpation assessment)

[7] Neck circumferences taken at 0.25, 0.5 and 0.75 neck length (measured from poll to highest point of the wither)

Table 5: Association of body fat measurements with BCS change and percent weight loss

Variable		Day						P-Value: Change in BCS	P-Value: Percent Weight Loss
		0	14	28	42	70	98		
Weight (kg)	Mean	548.4	546.2	534.2	530.1	509.8	501.4	.	.
	SEM	28.7	29.2	26.8	27.2	23.2	27.8		
Rump Fat Thickness (mm)[1]	Mean	11.1	11.3	10.4	9.5	7.1	6.1	<0.0001	<0.0001
	SEM	1.5	1.3	1.2	1.3	1.0	1.1		
Percent Fat[2]	Mean	8.52	8.62	8.15	7.87	6.33	5.83	<0.0001	<0.0001
	SEM	0.79	0.71	0.64	0.69	0.55	0.58		
Fat Mass (kg)[3]	Mean	46.7	46.9	42.7	40.9	32.0	29.1	<0.0001	<0.0001
	SEM	4.4	4.3	1.9	2.6	2.7	3.1		
Fat Free Mass (kg)[4]	Mean	502.0	499.3	491.5	489.1	477.8	472.3	0.002	<0.0001
	SEM	27.1	26.8	26.9	26.9	22.5	26.5		

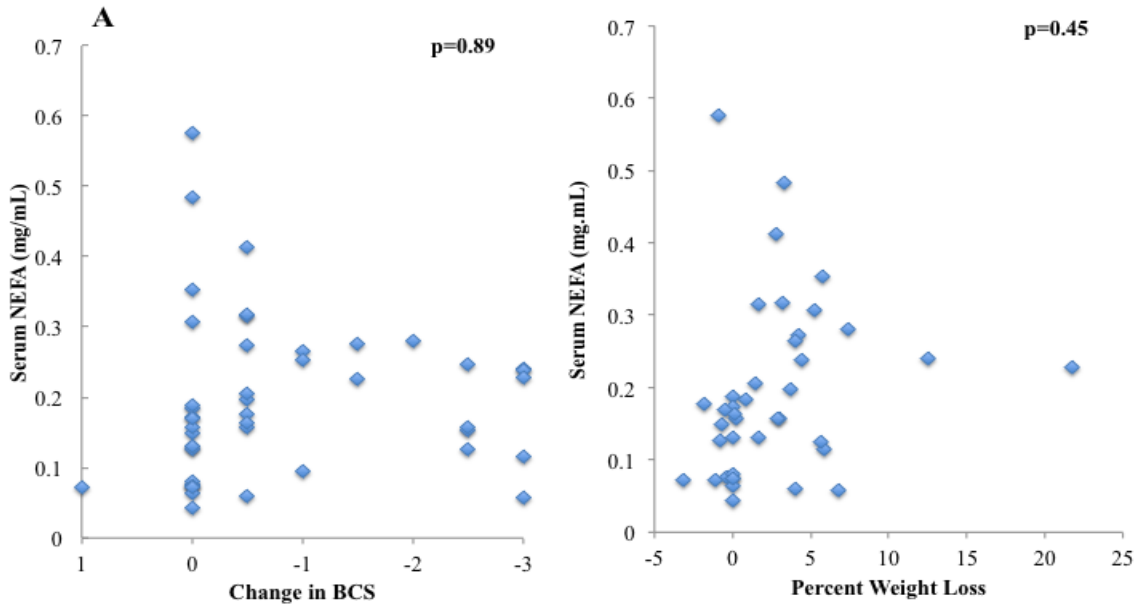
[1] Measured by B-mode ultrasound, site determined by measuring half the distance from the point of the hip (tuber coxae) to the point of the buttock (ischiatic tuberosity); and half of the distance from this point to the spine, units expressed in cm

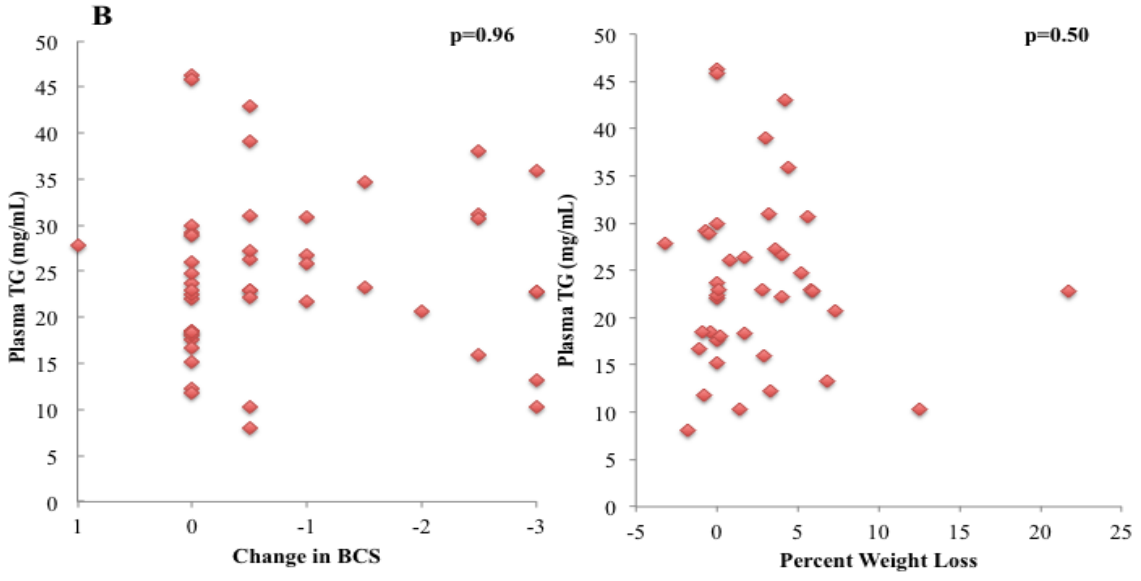
[2] Percent fat = 2.47 + (5.47 x rump fat thickness in cm)

[3] Fat mass = percent fat x body weight

[4] Fat free mass = body weight – fat mass

Figure 1 A-D: Effects of decreasing body condition and percent weight loss on circulating metabolic indicators. Horses (n = 8) were enrolled in a baseline-sampling period lasting from d0 to d14, followed by a caloric restriction from d15 to d98, where from d15 to d43 horses received a 30% digestible energy reduction, and from d44 to d 98 horses received a 30% restriction from maintenance digestible energy. Non-fasting blood samples were taken on d 0, 14, 21, 28, 35, 42, 56, 70, and 98 and were analyzed for serum non-esterified fatty acids (NEFA) and insulin, along with plasma triacylglycerides (TG) and glucose concentrations. The main effect of change in body condition score (BCS) and percent weight loss were analyzed for each variable, with data points representing values at corresponding points of BCS change or weight loss, respectively.





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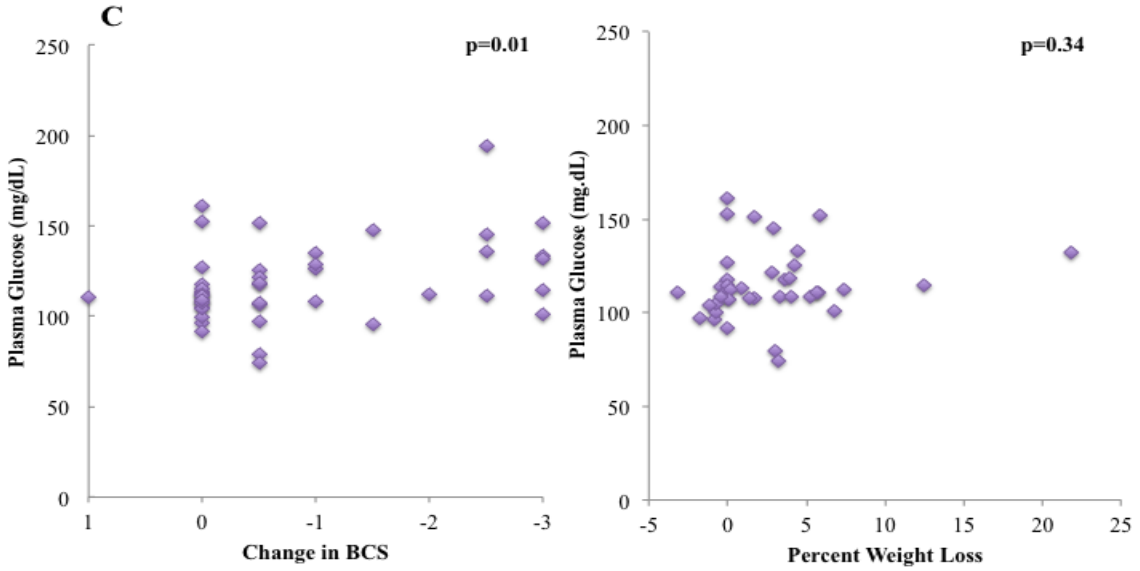


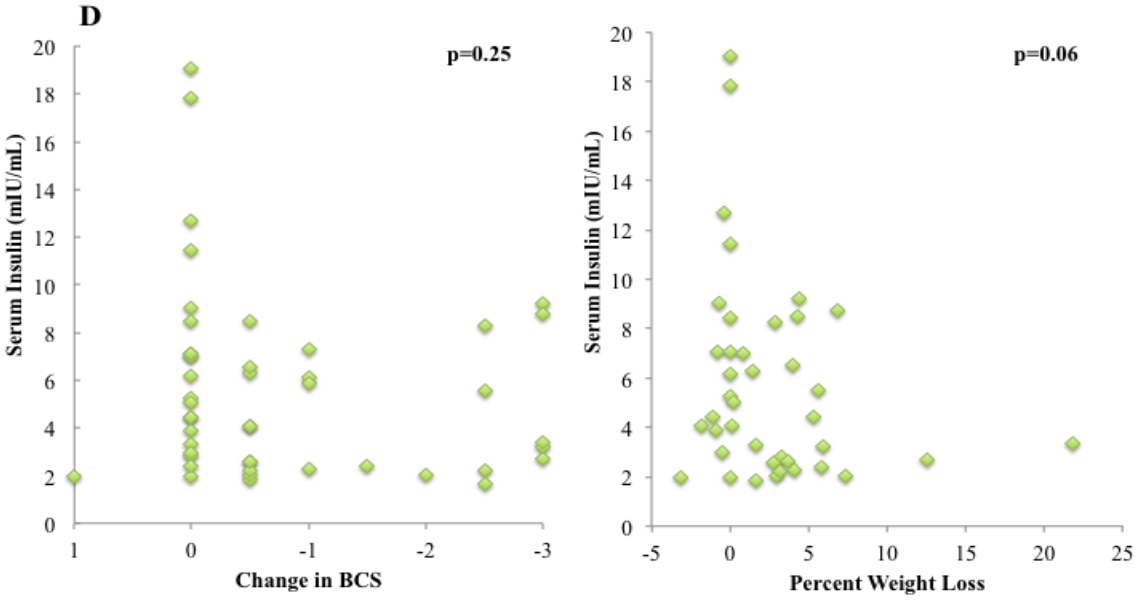
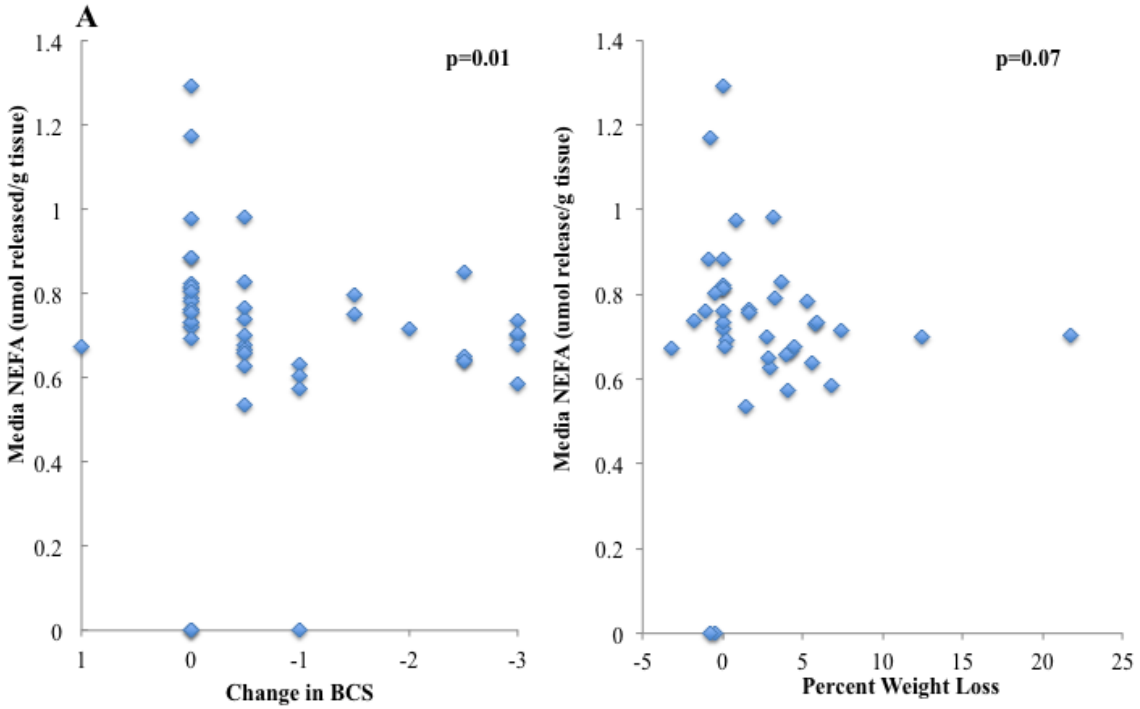


Figure 2 A-B: Influence of weight loss on lipolysis markers. Horses (n = 8) were enrolled in a baseline-sampling period lasting from d0 to d14, followed by a caloric restriction from d15 to d98, where from d15 to d43 horses received a 30% digestible energy reduction, and from d44 to d 98 horses received a 30% restriction from maintenance digestible energy. On d 0, 14, 28, 42, 70, and 98, a neck fat biopsy was collected and immediately allocated for an ex vivo lipolysis assay. Media collected from the assay was analyzed for non-esterified fatty acids (NEFA) and free glycerol concentrations. The main effect of change in body condition score (BCS) and percent weight loss were analyzed for each variable, with data points representing values at corresponding points of BCS change or weight loss, respectively.



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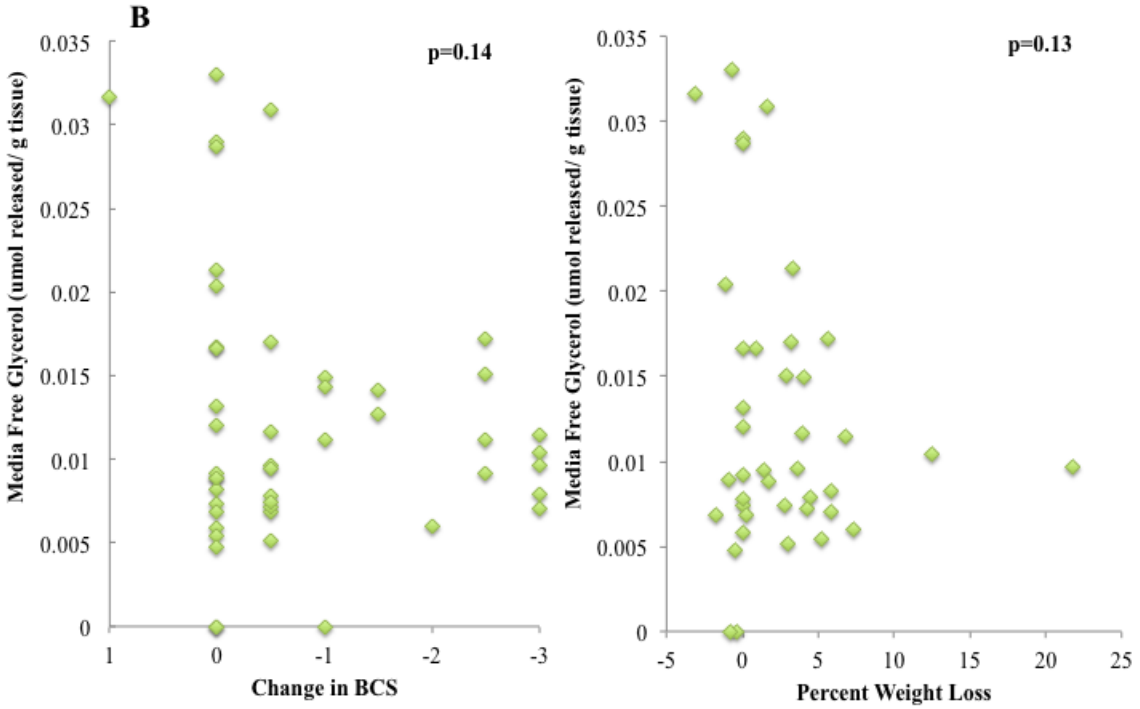
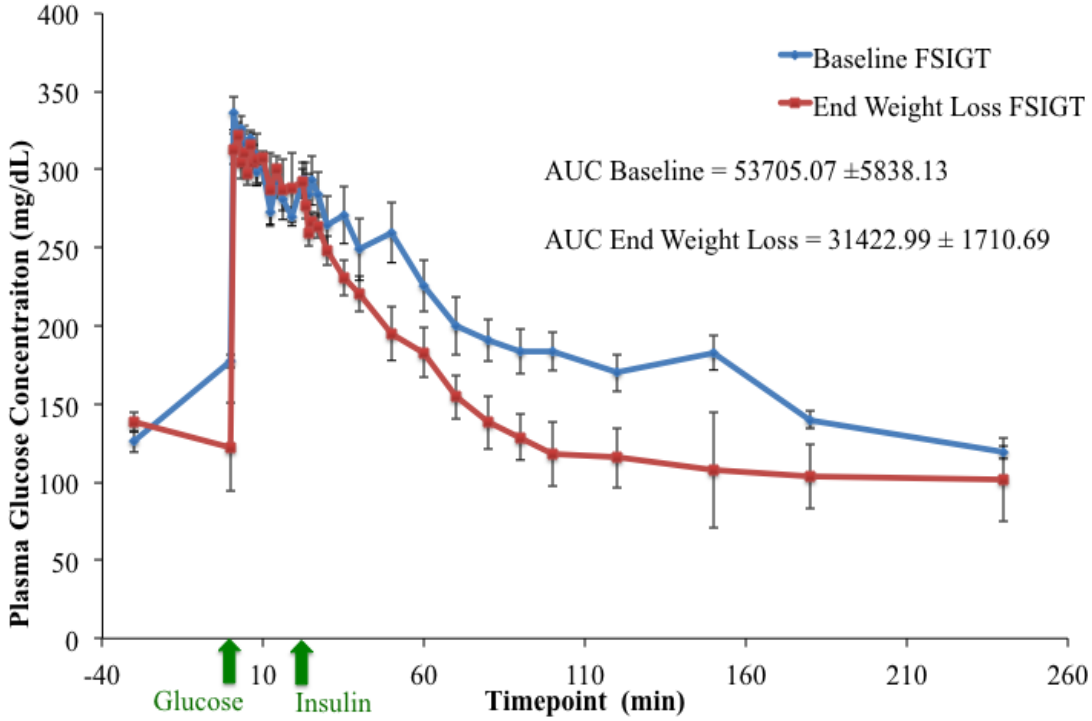


Figure 3 A-B: Glucose and insulin curves during baseline and end weight loss FSIGT. Horses (n = 8) were enrolled in a baseline-sampling period lasting from d0 to d14, followed by a caloric restriction from d15 to d98, where from d15 to d43 horses received a 30% digestible energy reduction, and from d44 to d 98 horses received a 30% restriction from maintenance digestible energy. On d 12-13 for the baseline sampling period and d 94-95 for the weight loss period a frequently sampled intravenous tolerance test (FSIGT) was administered to each horse in accordance with published methods (Hoffman et al., 2003). A glucose bolus (0.3 g/kg BW, 50% dextrose solution) was given at time point 0, followed by an exogenous insulin bolus (30 mIU/ kg BW, Humulin R) administered at minute 20, indicated by the green arrows. Blood samples were taken at -30, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 min post glucose administration and were analyzed for plasma glucose and serum insulin. Area under the curve was calculated using the trapezoidal method. The main effect of change in body condition score (BCS) and percent weight loss were analyzed for each variable, with data points representing values at corresponding points of BCS change or weight loss, respectively.

A



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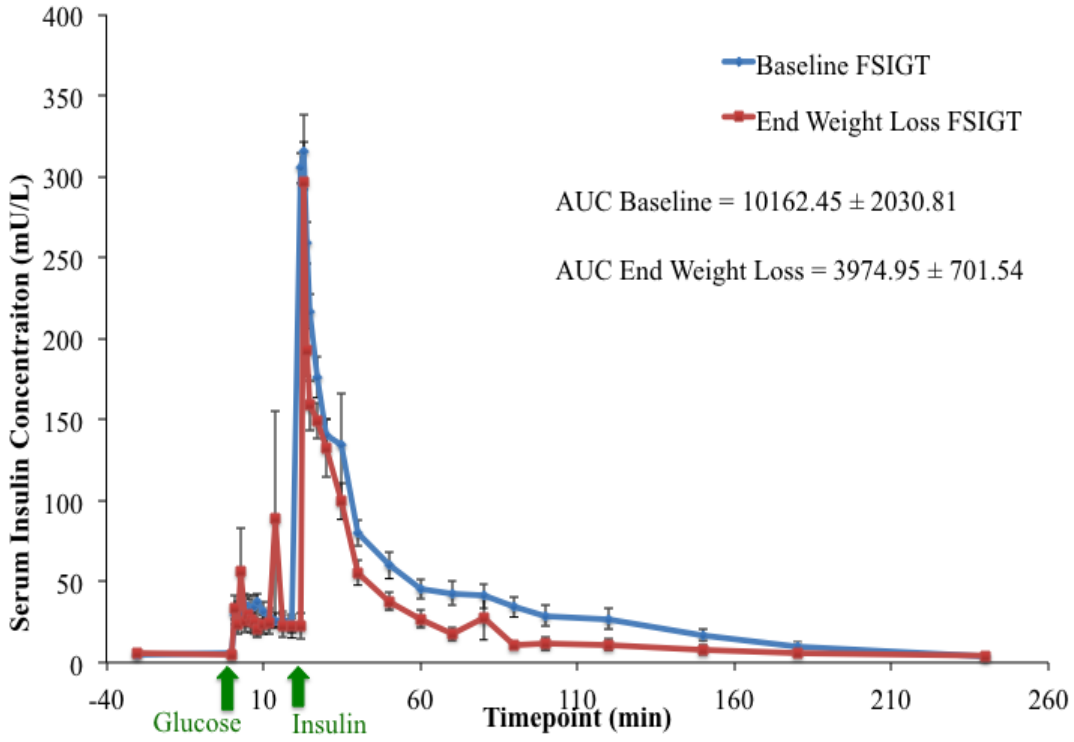

Figure 4 A-B: Representative illustrations of glucose (A) and insulin (B) curves during FSIGT by horse and date. Horses (n = 8) were enrolled in a baseline-sampling period lasting from d0 to d14, followed by a caloric restriction from d15 to d98, where from d15 to d43 horses received a 30% digestible energy reduction, and from d44 to d 98 horses received a 30% restriction from maintenance digestible energy. On d 12-13 for the baseline sampling period (Date 1) and d 94-95 for the weight loss period (Date 2) a frequently sampled intravenous tolerance test (FSIGT) was administered to each horse in accordance with published methods (Hoffman et al., 2003). A glucose bolus (0.3 g/kg BW, 50% dextrose solution) was given at time point 0, followed by an exogenous insulin bolus (30 mIU/ kg BW, Humulin R) administered at minute 20. Blood samples were taken at -30, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150 and 180 min post glucose administration and were analyzed for plasma glucose and serum insulin. Horses are numbered 1 through 8, corresponding with the assignments described in table 1, in order to illustrate independent horse variability during the study.

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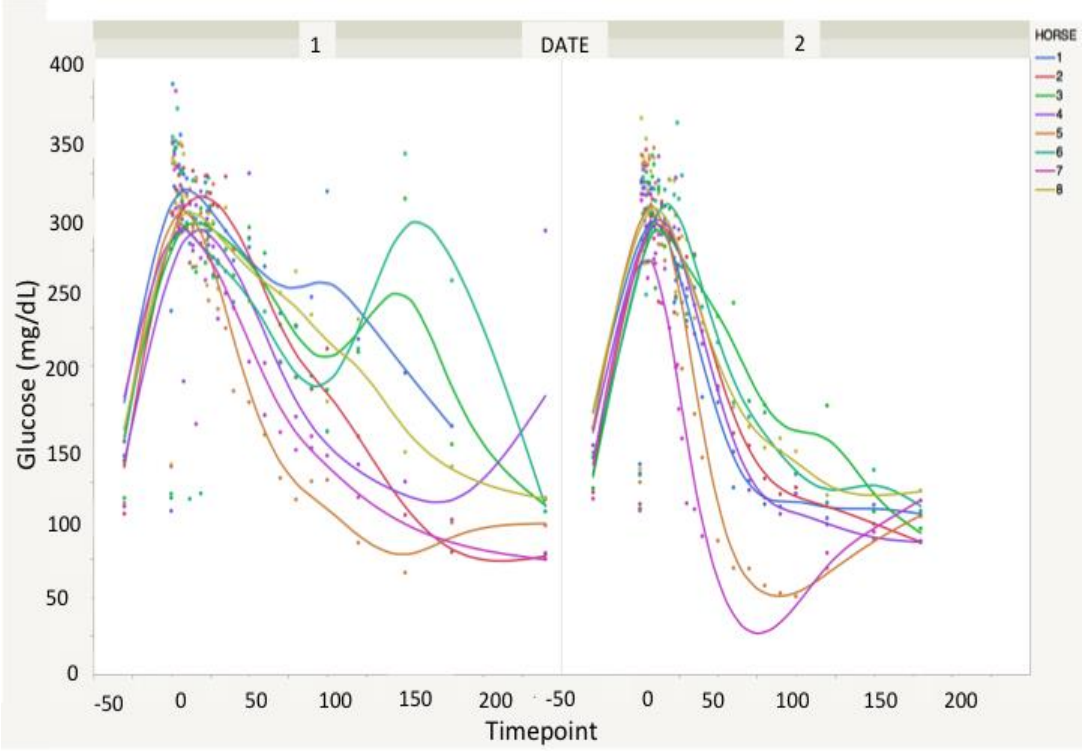

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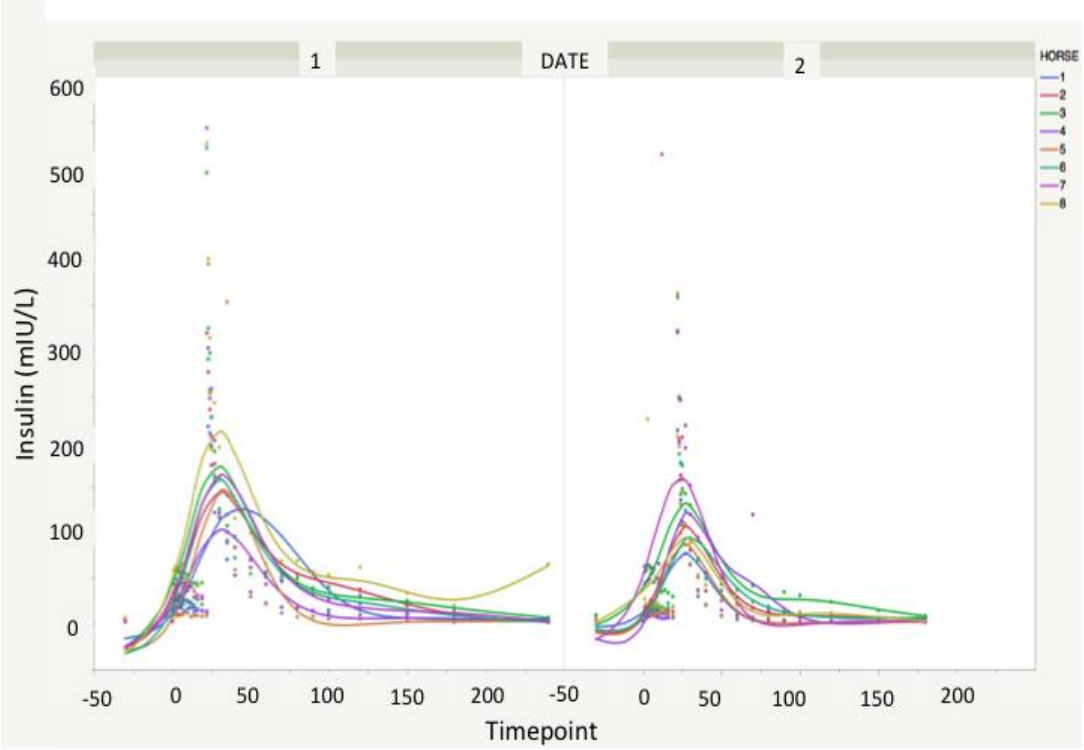

Table 6: Minimal model parameters during baseline sampling and at the end of weight loss.

Minimal Model Parameters[1]	End of Baseline Sampling Period[2,4]	End of Weight Loss Period[3,4]	p Value	
			BCS Change	Percent Weight Loss
GB (mg/dL)	159.9 ± 28.7	113.8 ± 4.5	0.07	0.003
IB (mU/mL)	4.51 ± 0.80	4.81 ± 0.43	0.53	0.96
G0 (mg/dL)	290 ± 18	329 ± 20	0.21	0.99
Sg (min-1)	0.0077 ± 0.0023	0.0234 ± 0.0110	0.41	0.81
Si (x10-4 L*mU-1*min-1)	1.078 ± 0.270	4.319 ± 1.592	0.04	0.01
AIRg (mU*min*L-1)	278.9 ± 57.6	217.0 ± 61.2	0.15	0.14
DI	260.17 ± 72.6	646.38 ± 213.86	0.19	0.09

[1] Calculated from FSIGT
Sg refers to glucose sensitivity
Si refers to insulin sensitivity
AIRg refers to acute insulin response to glucose
DI refers to the disposition index
GB refers to calculated baseline glucose concentration
IB refers to calculated baseline insulin concentration
G0 refers to highest concentration of glucose after bolus intravenous injection
[2] Sampled on d12 or d13
[3] Sampled on d 95 or d 96
[4] Mean ± SEM

Table 7: Correlations between morphometric measurements, body composition, circulating metabolic markers, and lipolysis data[1]

	Cresty Neck Score		Neck Circumference at 0.25 NL		Neck Circumference at 0.5 NL		Neck Circumference at 0.75 NL		Rump Fat Thickness		Percent Fat		Fat Mass		Fat Free Mass	
	R	P value	R	P value	R	P value	R	P value	R	P value	R	P value	R	P value	R	P value
Girth Circumference	-0.13	0.39	0.62	<0.0001	0.49	0.0004	0.52	0.0002	-0.12	0.43	-0.12	0.39	0.35	0.02	0.83	<0.0001
Abdominal Circumference	-0.27	0.08	0.49	0.001	0.37	0.01	0.44	0.0019	-0.12	0.41	-0.13	0.37	0.23	0.12	0.62	<0.0001
Neck Circumference at 0.25 NL	-0.002	0.98	.	.	0.70	<0.0001	0.76	<0.0001	0.03	0.82	0.03	0.86	0.48	0.0005	0.76	<0.0001
Neck Circumference at 0.50 NL	-0.14	0.92	.	.	.	.	0.85	<0.0001	0.01	0.93	0.01	0.92	0.42	0.0032	0.66	<0.0001
Neck Circumference at 0.75 NL	-0.08	0.59	.	.	.	.	.	.	0.07	0.65	0.05	0.71	0.48	0.0006	0.72	<0.0001
Neck Crest Height	0.4	0.0069	0.34	0.02	0.47	0.0010	0.51	<0.0001	0.48	0.0008	0.47	0.0011	0.58	<0.0001	0.18	0.21
Body Condition Score	0.77	<0.0001	0.24	0.20	0.28	0.06	0.19	0.19	0.56	<0.0001	0.57	<0.0001	0.63	<0.0001	0.09	0.55
Cresty Neck Score	.	.	0.01	0.99	0.01	0.92	0.08	0.59	0.63	<0.0001	0.62	<0.0001	0.50	0.0004	0.21	0.15
Liplysis Media NEFA	0.37	0.02	0.33	0.03	0.40	0.36	0.36	0.02	0.42	0.0050	0.42	0.0044	0.5	0.0005	0.18	0.22

[1]P values reflect significance and trends identified with bonferroni corrections, where significance (*) determined at $p \leq 0.0032$ and trends (#) noted when $p \leq 0.0047$.

CHAPTER III: JOURNAL ARTICLE II

CHANGES IN OXIDANT STATUS AND MITOCHONDRIAL FUNCTION IN RESPONSE TO WEIGHT LOSS IN AN OBESE HORSE MODEL

Abstract

Obesity causes a multitude of metabolic issues in the horse, yet stepwise alterations in mitochondrial capacity and oxidant status during weight loss have yet to be analyzed. Skeletal muscle contains subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria, which respond differently to physiological stimuli, impacting tissue and whole body oxidant status. We hypothesized that 8 light-type horses would display improvements in skeletal muscle mitochondrial subpopulation function and decreases in circulating oxidant status markers during weight loss from an obese (7 to 8) to moderate (5) body condition score (BCS). Effects of change in BCS or percent weight loss were evaluated using the mixed procedure of SAS with repeated measures. Plasma nitrate decreased in response to BCS reduction ($p=0.06$) and percent weight loss ($p=0.06$), whereas erythrocyte reduced glutathione ($p=0.06$) concentration increased with decreasing BCS. Mitochondrial electron transport chain complex I and IV displayed greater activity in SSM than IFM ($p\leq0.05$), while I, III, and IV in SSM had decreased activity due to BCS change and percent weight loss ($p\leq0.05$). Interactions between SSM and IFM complex IV activity and both weight loss markers ($p<0.05$) were displayed. Citrate synthase activity, indicating mitochondrial number, was greater in SSM than IFM ($p<0.0001$) but remained unchanged with weight loss parameters. Lipid peroxidation was decreased due to BCS change ($p=0.01$) and weight loss ($p=0.02$), with greater concentration in SSM, but a larger decrease in IFM ($p\leq0.05$). Alteration in complex activities and lipid peroxidation

suggest IFM are more affected by weight loss, with large contributions from complex IV byproducts. Mitochondrial component flexibility may contribute individually to development and disease propagation along with athletic performance of the equine athlete.

Key Words

Mitochondria, horse, weight loss, oxidant status, metabolism

Introduction

Within the United States, obesity among horses has reached an all time high, where nearly half of the nations horses are considered overweight or obese (Pleasant et al., 2008). Obesity-related metabolic changes are well known within the horse, including impairment of glucose tolerance and insulin signaling, which compounded with compromised lipid metabolism and mitochondrial function can cause increases in oxidative damage (Geor et al., 2007; Pleasant et al., 2008). While the effects of obesity on physiologic pathways have been studied, many areas still remain vague, especially with regard to the lasting effects of obesity on metabolic function and stepwise alterations in metabolic capacity during weight loss. Within the muscle, mitochondrial subpopulations may be differentially affected, but any impairment in function may give rise to increased reactive oxygen species through leaky electron transport chain complexes (Dabkowski et al., 2010). Elevated levels of oxidative damage can further propagate the aforementioned dysfunction associated with obesity (Muoio and Newgard, 2006). Due to these and other effects, weight loss is often the desired goal, ideally

translating a reduction in body condition into reversal of the impaired functions. However, most studies in which effects of obesity have been evaluated utilized two comparison groups; a lean or moderate and obese body condition models as opposed to evaluating the effects of weight loss within a specific individual. Therefore, this study was conducted to quantify the degree of oxidative damage and anti-oxidant defense along with identifying functional differences in mitochondrial subpopulations of skeletal muscle relative to body condition change and weight loss. We hypothesized that weight loss would decrease circulating levels of nitric oxide, while increasing glutathione and glutathione peroxidase levels. At the tissue level, lipid peroxidation would decrease corresponding to a decrease in body condition. Further, we suspected that subsarcolemmal mitochondria would show an increase in function, while interfibrillar mitochondria would remain relatively unchanged.

Materials and Methods

The Middle Tennessee State University Institutional Animal Care and Use Committee approved all methods and procedures used in this experiment. Horses were body condition scored by two individual, experienced reviewers and horses with assigned an body condition score (BCS) of 7 or greater (Henneke et al., 1983), were admitted to the study. In addition, horses chosen for this screening process were known to not have any predisposing metabolic conditions including Cushing's Disease or Metabolic Syndrome. Eight, healthy, mature (5-19 yrs) light type mixed sex (n=6 mares, n=2 geldings) horses of Quarter Horse, Tennessee Walking Horse, or Standardbred breeds from the Middle

692 Tennessee State University Horse Center herd were admitted for use in this study (Table
693 1).
694
695 *Sampling Periods and Diet*
696 Prior to and during the study, all horses received the same type of mixed grass hay and
697 commercial concentrate (Purina Strategy, Land O'Lakes Purina Mills, St. Louis, MO),
698 with *ad libitum* access to water. Dietary analysis was measured on concentrate samples
699 from 10 different bags and hay grab samples from at least 25 bales over 5 random dates
700 during the study (Equi-analyticial, Ithaca, NY, table 2 and 3). Further, study horses were
701 group housed in pastures with run-in shelters when not individually stalled over the entire
702 study. Pastures did contain some plant life; however, there was not enough coverage to
703 make a significant contribution to the diet and therefore was not included in DE
704 calculations or dietary analysis. Due to limiting labor factors, individual feed offerings
705 and refusals were not measured daily, but estimated based on average flake weight and
706 concentrate cup weight, taken from 10 total measurements.
707
708 The baseline sampling period (BSP) began on d0 and continued until d14, during which
709 time horses were maintained on their current dietary intake, with a mean DE intake of
710 21.1 Mcal/d. Upon entry into the weight loss period (WLP, d15), horses received a 30%
711 DE reduction by altering amount of concentrate and hay offered. Horses remained on this
712 level of restriction until d43, at which time calculations were made for maintenance DE
713 requirements based on current body weight, as described by the equation (NRC, 2007)
714 DE (Mcal/d) = 1.4 + (0.03 x BW). In order to facilitate further weight loss, a 30%

715 reduction from the maintenance requirement was calculated and implemented throughout
716 the remainder of the study (mean DE intake 14.01). At no point during the study did
717 horses consume less than 1.5% BW in forage per day to ensure proper hindgut health.
718
719 During both the BSP and WLP, all horses were subjected to exercise not exceeding the
720 parameters of light workload as defined by the NRC. Horses were exercised on average
721 between 1-3 hr/wk, with an approximate gait breakdown of 40% walk, 50% trot and 10%
722 canter. Horses not broke to ride were lunged or worked in a round pen.
723
724 *Sample Acquisition and Analysis*
725 Morphometric Measurements and Body Condition Score
726 Two experienced individuals assigned a body condition score (BCS) based on a 1-9 scale
727 (Henneke et al., 1983). Scores were assigned on a whole or half score basis and the
728 average was used for statistical analysis. Morphometric measurements consisted of body
729 weight, wither height, body length, girth circumference, and abdominal circumference, in
730 accordance with the measurements made by Carter and colleagues (Carter et al., 2009).
731 Measurements and BCS were made on d 0, 14, 28, 42, 70, and 98. Body length was
732 measured from the intermediate tubercale of the humerous (point of the shoulder) to the
733 ischiatic tuberosity (point of the buttock). Girth circumference was measured
734 immediately behind the slope of the wither, caudal to the elbow (olecranon tuber).
735 Abdominal circumference was taken at two-thirds the distance from the intermediate
736 tubercale of the humerous (point of the shoulder) the point of the hip (tuber coxae).

737 Horses were clipped in the areas where measurements were made initially to ensure later
738 measurements were taken at the same location.
739
740 Body Composition Measurements
741 Rump fat thickness was measured using B-mode ultrasound (Sonovet 2000, 5mgHz)
742 (Kearns et al., 2006a; Kearns et al., 2006b) on d 0, 14, 28, 42, 70, and 98. The site for
743 measurement was determined by measuring half the distance from the point of the hip
744 (tuber coxae) to the point of the buttock (ischiatic tuberosity); and half of the distance
745 from this point to the spine. Scans were made on alternating sides so as not to be affected
746 from healing biopsy sites. Body fat percentage was estimated using the following
747 equation (Kane et al., 1987); Percent Fat = 2.47 + 5.47 (rump fat in cm). Fat mass was
748 calculated by multiplying percent fat and total body mass. Fat free mass was determined
749 by the difference between total body mass and fat mass.
750
751 Circulating Oxidant Status Biomarkers
752 Non-fasting blood samples (~35 mL) were collected via jugular venipuncture on d 0, 14,
753 21, 28, 35, 42, 56, 70, and 98, placed into sodium heparin and EDTA vacutainers
754 (Vacutainer, Franklin Lakes, NJ). Tubes were immediately placed on ice. The EDTA
755 tube was centrifuged at 3000 x g for 15 min, while the sodium heparin tube was kept
756 aside and used for erythrocyte isolation. Plasma aliquots from the EDTA tube were
757 removed and stored at -20° C until analysis.
758

Sodium heparin plasma aliquots were used for nitric oxide approximation via the measurement of total nitrate by a colorimetric kit (Nitric Oxide Kit, Bioassay Systems, Hayward, CA) including deproteination for all plasma samples following the kit instructions. Duplicate coefficient of variation was accepted as 10% or below.

Erythrocytes were isolated following a protocol adapted from Lamprecht and Williams (Lamprecht and Williams, 2012). Briefly, 500 µL whole blood from a sodium heparin tube was transferred into a microcentrifuge tube, spun at 2500 x g for 5 min at 4° C, and the plasma supernatant was removed. Erythrocytes were then washed with 500 µL of 0.9% sodium chloride solution and centrifuged again at the same speed, duration and temperature. The supernatant was discarded and 1 mL ice-cold distilled deionized water was added to lyse the cells. Erythrocyte lysate was stored at -80° C until analysis for reduced glutathione (GSSH) and glutathione peroxidase-1 (GPx-1). Both GSSH and GPx-1 were analyzed using commercially available kits (GSH-400 and GPx-340, Oxis Biomedical Research, Foster City, CA). Protocols on the kit insert were followed and sample volumes adjusted to produce repeatable results within the detectable range. The accepted coefficient of variation between duplicates was 10% for both assays.

Muscle Biopsies

Muscle biopsies were collected on d 0, 14, 28, 42, 70, and 98. Prior to biopsy sampling, biopsy sites were measured, clipped, and scrubbed with chlorhexadine solution followed by an isopropyl alcohol rinse. Horses were then lightly sedated with xylazine (0.5mg/kg

781 BW) and were administered 10 cc lidocaine anesthesia to the rump site. Consecutive
782 samples were taken on alternating sides so as to allow sites to fully heal.
783
784 Muscle biopsy site measurements were calculated by determining half the distance from
785 the point of the hip (tuber coxae) to the point of the buttock (ischiatic tuberosity). From
786 this point, a measurement was made to the spine, and biopsy site was placed at half this
787 length, with preparations made as stated above (Andrews et al., 1993). A Bergstrom
788 biopsy needle (6mm diameter, 10 cm total length) was used to access the middle gluteal
789 muscle (Kline et al., 1987; Liburt et al., 2012), where 1 g of tissue was removed. Biopsy
790 sites were stapled closed, and left to heal for 10 d. Weighed sample allocations were
791 made for immediate mitochondrial isolation.
792
793 Mitochondrial Subpopulation Isolation
794 Muscle biopsies were blotted dry, weighed into 100 mg pieces and rinsed with ice-cold
795 Chappel-Perry buffer in preparation for SSM and IFM isolation. Methods were followed
796 in accordance to those used by Palmer and colleagues (Palmer et al., 1977b) with minor
797 modifications (Dabkowski et al., 2010; Dabkowski et al., 2009; Dabkowski et al., 2008b).
798 Briefly, the samples were homogenized 1:10 (wt/vol) in ice-cold Chappel-Perry buffer,
799 containing (in mmol/L) 100 KCl, 50 MOPS, 5 $MgSO_4 \bullet 7H_2O$, 1 EGTA, and 1ATP at pH
800 7.4. Homogenates were centrifuged at 700 x g for 10 min and supernatant containing
801 SSM was removed and centrifuged again at 10,000 x g for 10 min to yield the SSM
802 fraction. A series of rinse and centrifugation at 10,000 x g for 10 min were conducted to
803 obtain a clean SSM fraction, resuspended in 100 uL KCl-MOPS-EGTA buffer. The

804 remaining pellet from the 700 x g spin was resuspended in KCl-MOPS-EGTA buffer
805 containing, in mmol/L, 100 KCl, 50 MOPS and 0.5 EGTA at pH 7.4 and exposed to 3.75
806 mg/g trypsin for 7 min. After 7 min, the resuspended IFM were diluted twofold with
807 buffer plus protease inhibitor cocktail (Biovision, Mountain View, CA) to inhibit further
808 trypsin activity and centrifuged at 700 x g for 10 min. The IFM-supernatant was saved
809 while the remaining pellet was resuspended in KCl-MOPS-EGTA buffer and spun down
810 again at 700 x g for 10 min to maximize IFM yield. The IFM supernatants were
811 combined and spun down at 10,000 x g for 10 min. After a series of wash steps, IFM
812 were spun down at 10,000 x g for 10 min and the resulting pellet was resuspended in 100
813 uL KCl-MOPS-EGTA buffer. Both populations were stored at -80° C until analysis.
814
815 Complex Activities and ATP Synthase
816 Electron transport chain complexes I, III, and IV activities were measured
817 spectrophotometrically (Biotek Synergy HT plate reader, Biotek, Winooski, VT) on
818 isolated frozen-thawed mitochondria as described by Dabkowski and colleagues
819 (Dabkowski et al., 2010). Complex I activity was measured by the oxidation of NADH in
820 the presence of an assay mixture containing 25 mM potassium phosphate buffer (pH 7.2),
821 5mM $MgCl_2$, 2mM KCN, 2.5 mg/mL bovine serum albumin, 50 μM NADH, 10 μM
822 decylubiquinone, and 2 μg/mL antimycin A at 340 nm. The reaction was initiated at the
823 addition of isolated skeletal muscle mitochondrial subpopulations, and enzyme activity
824 was measured every 10s for 3 min. Complex I specific activity was inhibited by 2 μg/ mL
825 rotenone. Complex III activity was quantified by measuring the reduction of cytochrome
826 c at 550 nm. An assay buffer containing 2mM EDTA, 500 mM sucrose, 100 mM

827 Tris·HCl (pH 7.4), 1mM cytochrome c, 1 mg/mL antimycin A and reduced
828 decycloubiquinone was combined with mitochondrial isolations and spectrophotometric
829 measurements were made in 10 s increments for 3 min. Lastly, complex IV was measured
830 by adding an assay mixture of 10 mM phosphate buffer (pH 7.4) and 20 μM reduced
831 cytochrome c, where readings at 550 nm every 10 s for 3 min began at the addition of
832 mitochondrial isolate determined oxidation of cytochrome c. Protein content was
833 determined via the Bradford assay (Bradford, 1976) using bovine serum albumin as the
834 standard. Values for complex activity data are expressed as nmol substrate consumed per
835 min per mg protein.
836
837 ATP synthase activity was measured via approximation of maximal hydrolytic capacity
838 of the enzyme in accordance with previously published methods (Dabkowski et al., 2010;
839 Feniouk et al., 2007; Pullman et al., 1960; Rosca et al., 2009). Activity was measured
840 spectrophotometrically via a coupled assay with lactate dehydrogenase and pyruvate
841 kinase as coupling enzymes on frozen-thawed mitochondria as oligomycin-sensitive
842 ATP-ase activity (Barrientos, 2002). Briefly, the mitochondria were incubated with a
843 buffer containing, in mmol/L, 20 HEPES, 5 MgCl2, 100 KCl, 2.5 phosphoenolpyruvate,
844 and 0.2 NADH, along with 0.1 mg/mL pyruvate kinase and 0.1 mg/mL lactate
845 dehydrogenase, at combined pH of 7.5-8.0. The reaction was initiated by adding ATP to
846 a final concentration of 1 mM and followed by NADH reduction at 340 nm. Absorbance
847 was monitored in 10 s increments for 3 min using a Biotek Synergy HT plate reader
848 (Biotek, Winooski, VT). Protein content was measured as described above, with data

849 expressed as μmol consumed per min per mg protein, which is equal to nmol of NADH
850 oxidized per min per mg of protein.
851
852 Citrate Synthase
853 Citrate synthase activity was measured on frozen-thawed mitochondria utilizing the
854 reaction 5', 5'-Dithiobis 2-nitrobenzoic acid (DTNB) and CoA-SH to form TNB, as
855 instructed by a commercially available kit (Citrate Synthase Activity, ScienCell Research
856 Labroatories, Carlsbad, CA) (Morgunov and Srere, 1998; Trounce et al., 1996). The
857 intensity of the absorbance of the assay at 412 nm is proportional to citrate synthase
858 activity in the sample. As the assay was not completed on a fresh isolation, citrate
859 synthase activity is used as a measure of total mitochondrial population in each isolation,
860 rather than cleanliness of the isolation procedure. Data are expressed as activity/mg
861 mitochondria. Mitochondrial content was measured by protein concentration assayed and
862 calculated as stated above.
863
864 Lipid Quantification and Peroxidation
865 In order to estimate total lipid presence in mitochondrial isolations, a sulfo-phospho-
866 vanillin total lipid quantification procedure was conduced in accordance to previously
867 published methods (Cheng et al., 2011). To summarize, each sample was diluted in a 2:1
868 (v/v) chloroform:methanol mixture and allowed to fully evaporate. At this point, 100 μL
869 concentrated sulfuric acid was added to each well, incubated at 90° C for 20 min, and
870 allowed to cool at room temperature before reading for background absorbance at 540 nm
871 (Biotek Synergy HT plate reader, Biotek, Winooski, VT). Then, 50 μL vanillin and

phosphoric acid mixture (0.2 mg/mL Vanillin in 17% phosphoric acid) were then added to each well and allowed to develop for 10 min before a final absorbance was read at the 540 nm. The difference between the two readings was calculated and sample concentrations were calculated from a standard curve in μg/μL.

Lipid peroxidation was assessed by the measurement of two stable end products, malondialdehyde (MDA) and 4-hydroxyalkenal (4-HAE), formed from the oxidation of polyunsaturated esters and fatty acids (Dabkowski et al., 2009; Dabkowski et al., 2008a). Frozen-thawed mitochondrial subpopulation isolations were analyzed for MDA and 4-HAE using a commercially available colorimetric kit (Oxford Biomedical Research, Oxford, MI). In brief, this assay is conducted based upon the reaction of a chromogenic reagent, N-methyl-2-phenylindole, reagent 1, with MDA and 4-HAE at 45° C. One molecule of either MDA or 4-HAE reacts with one molecule of reagent 1 to yield a stable chromophore, detectable at 586 nm. Absorbance was measured on a Biotek Synergy HT plate reader (Biotek, Winooski, VT), where protein concentration allowed for equal sample loading. Final values are expressed per μg/μL total mitochondrial lipid.

Statistical Analysis

Data are expressed as mean ± SEM. Normality of all data were assessed prior to statistical analysis (SAS Institue, V9.3, Cary, NC). Any variable found to be non-normally distributed by evaluation of kurtosis and skewness values, along with significance in tests for location (students t and sign tests) and normality (Shapiro-Wilk and Klomogorov-Smirinov tests) where $p \leq 0.05$, were $\log_{10}$ transformed. The effect of

BCS change and percent weight loss was tested for all data using proc MIXED procedure of SAS with repeated measures. Gender, horse, age and date were included in the class statement during repeated measures analysis. Similarly, when evaluating complex I, III, IV, ATP synthase, citrate synthase and activities, along with lipid peroxidation, subpopulation was also included in the model and class. The model with the best fit according to Akaike's Information Criterion used a compound symmetry structure. A Pearsons Correlation was run using the proc CORR procedure of SAS, with Bonferroni correction to prevent the incidence of type I errors when performing multiple comparisons. For all data except correlations $p<0.05$ was considered significant and trends were noted at $p<0.10$. Due to the bonferroni correction, $p<0.0021$ was considered significant and trends were noted when $p<0.0052$ for all correlations.

Results

Body and Morphometric Measurements

Horses on the study transitioned from a mean BCS of 7.8 ± 0.02 a moderate body condition, 5.0 ± 0.0 during the course of the weight loss period. A summary of horse age, breed, sex and body characteristics are reported in table 1. Averages of body weight, girth circumference, abdominal circumference, RFT, percent fat, fat mass and fat free mass are reported in table 4.

Circulating Oxidant Status Markers

In relationship to change in body condition score, trends toward significance were displayed for decreasing nitrate concentration ($p=0.06$) and increasing reduced

glutathione concentration (p=0.07), while no differences were noted with regard to GPx (figure 1). When analyzed in response to percent weight loss, glutathione and GPx were not significant but nitrate concentration retained a trend (p=0.06, figure 1 A-C).

Electron Transport Chain Enzyme Activities, Citrate Synthase and Lipid Peroxidation

Significant differences were found between mitochondrial subpopulations regarding decreasing complex I, and IV activity, while a trend was observed for complex III activity, where p=0.02, 0.001, and 0.06, respectively. Further, activity in complex I (p=0.01), III (p<0.0001) and IV (p=0.001) decreased in response to change in BCS. The interaction between mitochondrial subpopulation and change in body condition score was significant for complex IV (p=0.001), but not complexes I or III where IFM decreased with change in BCS but SSM did not. ATP synthase activity was not found to be significantly affected for any comparison or interaction. Data analysis for complexes I, III and IV in addition to ATP synthase are represented in figure 2 A-D.

When complex enzyme activities were analyzed with respect to percent weight loss, complex I displayed a decrease in activity with regard to mitochondrial subpopulation (p=0.03) and percent weight loss (p=0.002), but no effect was denoted for the interaction (Figure 2A). Complex III displayed a significant reduction in activity in response to weight loss (p<0.0001), and a trend for differences between subpopulations (p=0.06) yet the interaction was not significant (Figure 2B). Subpopulation (p=0.003) and weight loss (p=0.01) caused a decrease in complex IV activity, further displayed by an interaction between these parameters (p=0.05, Figure 2C), signifying differential effects of weight

941 loss parameters on each subpopulation within this complex. No differences were found
942 with regard to ATP synthase activity and any included model parameter (Figure 2D).
943
944 As an approximate measure of mitochondrial number, citrate synthase activity was found
945 to be different between subpopulations ($p<0.0001$) in both models where SSM showed a
946 higher concentration than IFM, but no effect of change in body condition score, percent
947 weight loss or appropriate interaction was observed (Figure 3).
948
949 A significant effect of subpopulation ($p=0.01$) was observed where SSM displayed
950 greater amounts of lipid peroxidation than IFM. Change in body condition and
951 interactions between model components were observed for lipid peroxidation ($p=0.01$,
952 0.004, and 0.05, respectively) where peroxidation levels decreased due to reduction in
953 BCS. Similarly, when evaluated in response to weight loss, significance was observed
954 between higher levels of peroxidation in the SSM and IFM mitochondrial subpopulations
955 ($p=0.02$), and decreases in peroxidation due to percent weight loss ($p=0.01$), with a trend
956 for significance displayed for the interaction ($p=0.07$ Figure 4).
957
958 *Correlations*
959 Body condition score was positively correlated to lipid peroxidation in the IFM
960 ($p=0.002$). Complex III activity in the SSM ($p<0.0001$) and complex IV ($p=0.0002$)
961 activity in the IFM showed a significant positive correlation to BCS. Similarly when
962 correlating these variables to cresty neck score, significance was determined between

SSM complex III activity (p=0.002) and a trend was noted between IFM complex IV activity (p=0.003).

Citrate synthase activity in the SSM was negatively correlated to complex IV in the same subpopulation (p=0.002). Further, citrate synthase activity between the subpopulations displayed a significant positive correlation (p<0.0001). Interfibrillar citrate synthase activity was significantly correlated to lipid peroxidation within the subpopulation (p<0.0001). A summary of these and other correlation data can be found in table 5.

Discussion

Body composition is an important factor to consider when evaluating metabolic function, specifically with regard to glucose and lipid metabolism (Pedersen et al., 2003; Reaven, 1995). Due to the subjective nature of the Henneke body condition scoring system along with wanting to examine changes in regional adiposity, we included morphometric measurements to provide a comprehensive picture of phenotypic body adipose deposits (Carter et al., 2009; Henneke et al., 1983); however, body composition can be challenging to measure in a live animal. The concurrent reduction in rump fat thickness with decreasing body weight suggests horses were loosing fat mass as they transitioned to a moderate body condition. Further, the reduction in fat free mass suggests that some portion of lean body tissues were utilized for energy homeostasis, consistent with metabolic processes during calorie restriction. Although horses were enrolled in a light exercise program during the time of the study, horses were acclimated to this level of exercise; therefore, no skeletal muscle adaptation to workload was expected.

Skeletal muscle mitochondrial distribution is directly related to fiber type composition and therefore responses to physiological states can be altered depending on muscle fiber profile. In humans, metabolic disturbances associated with obesity and diabetes are associated with low percentages of type I fibers (Hickey et al., 1995). Further, mice engineered to have a high number of type I fibers are more insulin sensitive and less susceptible to diet-induced obesity (Ryder et al., 2003). The middle gluteal muscle sampled during this study for mitochondrial evaluation reflects a mixed fiber type, where previous research shows at a depth of 6 cm type I slow oxidative fibers represent 26.7 ± 5.9%, type IIa fast oxidative fibers characterize 54.9 ± 8.3% and type IIb fast glycolytic fibers comprise 18.4 ± 8.4%, respectively (Kline et al., 1987). Therefore, we expect the mitochondria isolated and analyzed in this study should reflect a mixed population and role within functioning muscle. The distribution signifies an equal ability to tolerate both aerobic and anaerobic respiration along with various energy substrates, thereby incorporating representative differences in weight loss across associated skeletal muscle fiber responses

Although it is recognized that skeletal muscle mitochondria exist together in a reticulum, compartmentalization into SSM and IFM allow for differential effects of physiologic intervention or disease states, and thus should be studied independently (Palmer et al., 1977a). For example, in mice subjected to streptozocin-induced type I diabetic insult, cardiomyocytes displayed greater IFM than SSM dysfunction, whereas in a db/db type 2 diabetic model the SSM was more affected (Baseler et al., 2011; Dabkowski et al., 2010).

The SSM provide energy for membrane related processes including signal transduction, ion exchange, and substrate transport and substrate activation (Hood, 2001); metabolic processes necessary for insulin signaling and glucose metabolism. The SSM evaluated in this study did not display a large change in activity, and based on the relatively conserved function of the SSM complex activities during weight loss, it is plausible that the SSM played a role in the improved insulin sensitivity displayed in the aforementioned chapter. As the SSM were not dysfunctional during weight loss, their function may have been important to the modulation of the obese phenotype, reflected by the lack of change in glucose mediated glucose disposal and other minimal model parameters. Currently, the only available equine-related information has utilized activity of mitochondrial marker enzymes such as succinate dehydrogenase or mitochondrial volume density to estimate muscle oxidative capacity (Hoppeler et al., 1987; Kayar et al., 1989; Quiroz-Rothe and Rivero, 2001; Snow and Guy, 1980), with no data describing the roles of subpopulations on overall energy generation. In human and rodent obesity models, the SSM is primarily affected, displaying greater levels of complex activities during weight loss or when obese models are compared to a lean counterpart (Kelley et al., 2002a; Ritov et al., 2005; Toledo et al., 2007). Previous work from our laboratory suggests equine skeletal muscle mitochondria subpopulations may play a more equal role than that of rodents (Zambito et al., 2013). The greater malleability of IFM function with relationship to complex I, III and IV activity suggests that within the horse, this subpopulation may be more sensitive to physiological states.

Further, results from this study display a decreasing maximal activity for complex I, III and IV in obese horses transitioning through weight loss. These results are contrary to most published research both relating to obesity-related, diabetic models, and weight loss influences on mitochondrial dysfunction in rodents and humans (Bajpeyi et al., 2011; Dabkowski et al., 2008a; Kelley et al., 2002b; Ritov et al., 2005; Toledo et al., 2007). Thus far, little work has been completed evaluating equine mitochondrial function (Votion et al., 2012) leaving many areas unclear with regard to species related differences in mitochondrial adaptation to physiological change. The higher level of activity when horses were at an obese body condition score may signify that the mitochondria compensated for excess dietary nutrient influx, and thus were the foundation for the lack of differences seen elsewhere. The flexibility of both populations of mitochondria, specifically the IFM, could not only be an evolutionary advantage for the horse to alter metabolic function in the face of differing nutritional status but also may tie into the inherent exercise capability of the horse over many other species.

In addition to their primary roles in substrate oxidation and ATP generation, mitochondria are also a primary source of reactive oxygen species. Declining complex activity should not necessarily be viewed as a negative result, as decreasing activity may reduce reactive oxygen species production. As body condition decreases and percent weight loss increases, nitrate levels as an indicator of nitric oxide decreases, while glutathione concentration increases. Combined these data suggest that overall whole body oxidant status was improving as horses lost weight. Glutathione peroxidase activity was

not altered due to weight loss, yet it is plausible that the level of oxidative stress was not elevated to the degree to impact enzyme activity.

Lipid peroxidation decreased in both SSM and IFM, with a greater level of damage observed in the SSM. As mentioned previously, electron transport chain complex I, III, and IV activity displayed a greater decrease in the IFM, thus generation of reactive species should decline in a similar manner. The resulting decrease in free radicals and reactive oxygen species would explain the decrease in lipid peroxidation observed in the IFM; a theory that could likewise be applied to the SSM. The higher level of lipid peroxidation observed in the SSM may be a function of mitochondrial number, as estimated by citrate synthase activity or position within the cell. Location directly beneath the plasma membrane may subject this population to oxidative insult from circulating oxidant species along with reactive oxygen species produced from the SSM alone.

Further, the possibility remains that due to high volume of oxidative phosphorylation capacity reflected by the nearly 50-fold increase from resting to maximal VO_2 (Poole, 2004), horses may have adapted to tolerate higher levels of oxidative stress. Splenic contraction is a unique performance advantage to the horse (Poole, 2004) and even in an obese horse model and from an evolutionary perspective they still must retain a certain level of athletic ability despite a greater fat mass. The ability to tolerate high oxygen concentrations in the muscle without excessive oxidative damage may prove to be beneficial in the instance of obesity-induced increases in oxidant status. Further, if the

mitochondria had increased function to cope with an excess substrate load, the reduction in activity would signify return to a normal level of operation. Comparison of basal metabolic rates between mean obese and moderate BCS weights display a reduction from 18 Mcal/d to 16 Mcal/d, as calculated by body weight $(kg)^{0.75}$ x 115 kcal. Decreased basal metabolic rate could also result in a lower complex activities and an overall reduction in oxidant status. Without supplemental data and analysis of the mechanisms behind mitochondrial function in the horse, exact understanding of these changes remains unanswered.

In conclusion, data suggest equine skeletal muscle mitochondrial subpopulations in this study contradict central dogma in human and rodent models regarding obesity- and weight loss-related influences on SSM and IFM function. The degree of obesity achieved prior to weight loss was adequate to induce increases in complex activity, and oxidant status within the body, yet not to the degree to transition into a metabolic disease state. Additional studies are warranted to not only elucidate mechanisms behind mitochondrial malleability in the equine model but also to fully determine the impact of mitochondrial function on exercise capacity.

Acknowledgements

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Tables and Figures

Table 1: Summary of individual horse characteristics

Horse	Age (yr)	Sex[1]	Breed	Wither Height (cm)[2]	Body Length (cm)[3]	Neck Length (cm)[4]
1	10	M	Quarter Horse	142.2	157.5	71.1
2	6	M	Tennessee Walker	148.0	137.2	71.1
3	13	M	Standardbred	160.0	156.2	101.6
4	19	G	Quarter Horse	149.9	149.9	86.4
5	5	M	Quarter Horse	147.3	152.4	81.3
6	10	G	Quarter Horse	170.8	165.1	96.5
7	19	M	Quarter Horse	151.8	158.8	95.3
8	15	M	Quarter Horse	157.5	165.1	104.1
			Mean ± SEM	153.4 ± 3.2	155.3 ± 3.3	88.4 ± 4.6

[1] For sex categorization M represents mare and G represents gelding.

[2] Measured from the ground to the highest point of the wither

[3] Measured from the point of the shoulder to the point of the rump

[4] Measured from the poll to the point of union between neck musculature and the wither

Table 2: Nutrient analysis of grass hay and concentrate, as fed

Nutrient, as fed	Grass Hay	Concentrate
Dry Matter (%)	95.8	93.4
Digestable Energy (DE, Mcal/kg)	1.85	2.84
Crude Protein (g/kg)	51.5	150.1
Acid Detergent Fiber (ADF, g/kg)	371.8	166
Neutral Detergent Fiber (NDF, g/kg)	625.8	330.8
Water Soluble Carbohydrates (WSC, g/kg)	56.0	89.4
Ethanol Soluble Carbohydrates (ESC, g/kg)	15.5	51.7
Starch (g/kg)	14.3	131.4
Non-fiber Carbohydrates (NFC, g/kg)	156.8	350.9
Crude Fat (g/kg)	28.6	59.3
Calcium (g/kg)	6.03	12.91
Phosphorus (g/kg)	0.83	8.15
Sodium (g/kg)	0.218	2.369

Table 3: Percent composition of diet on a dry matter basis

Nutrient, % dry matter	Grass Hay	Concentrate
Crude Protein, %	5.4	16.1
Acid Detergent Fiber, %	38.8	17.8
Neutral Detergent Fiber, %	65.4	35.4
Water Soluble Carbohydrates, %	5.9	9.6
Ethanol Soluble Carbohydrates, %	1.6	5.5
Starch, %	1.5	14.1
Non-fiber Carbohydrates, %	16.4	37.6
Crude Fat, %	3	6.3
Calcium, %	0.63	1.38
Phosphorus, %	0.09	0.87
Sodium, %	0.023	0.254

Table 4: Physical descriptors during baseline sampling compared to end of weight loss period

	Baseline Sampling Period[1]		End Weight Loss Period[2]	
	Mean	SEM	Mean	SEM
Weight (kg)	547.3	4.9	487.0	8.4
Body Condition Score[3]	7.8	0.02	5.0	0.00
Girth Circumference (cm)[4]	194.9	0.6	188.3	1.2
Abdominal Circumference (cm)[5]	213.6	0.7	199.7	1.9
Rump Fat Thickness (mm)[6]	11.2	0.2	5.3	0.2
Percent Fat[7]	8.6	0.1	5.3	0.1
Fat Mass (kg)[8]	46.7	0.7	27.3	0.9
Fat Free Mass (kg)[9]	500.6	4.6	456.9	8.1

[1] Reported measurements taken on d14 as horses exited the baseline sampling period

[2] Reported measurements taken on d 98 as horses exited the study

[3] Assessment based on a 1-9 scale (Henneke et al., 1983)

[4] Measured immediately behind slope of wither, caudal to elbow

[5] Measured at two thirds distance from point of shoulder to point of hip

[6] Measured by B-mode ultrasound, site determined by measuring half the distance from the point of the hip (tuber coxae) to the point of the buttock (ischiatic tuberosity); and half of the distance from this point to the spine, units expressed in cm

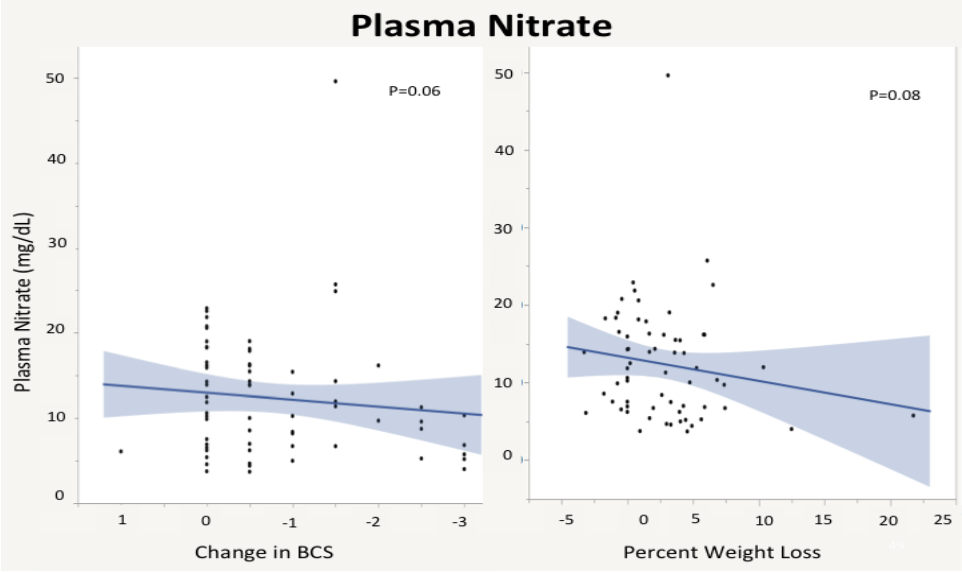
[7] Percent fat = 2.47 + (5.47 x rump fat thickness in cm)

[8] Fat mass = percent fat x body weight

[9] Fat free mass = body weight – fat mass

Figure 1 A-C: Changes in circulating oxidant status markers in relationship to change in body condition score and weight loss. Horses (n = 8) were enrolled in a baseline-sampling period lasting from d0 to d14, followed by a caloric restriction from d15 to d98, where from d15 to d43 horses received a 30% digestible energy reduction, and from d44 to d 98 horses received a 30% restriction from maintenance digestible energy. On d 0, 14, 21, 28, 42, 56, 70, and 98, blood samples were collected and analyzed for plasma nitrate concentration, erythrocyte glutathione concentration and erythrocyte glutathione peroxidase activity. The main effect of change in body condition score (BCS) and percent weight loss were analyzed for each variable, with data points representing values at corresponding points of BCS change or weight loss, respectively.

A



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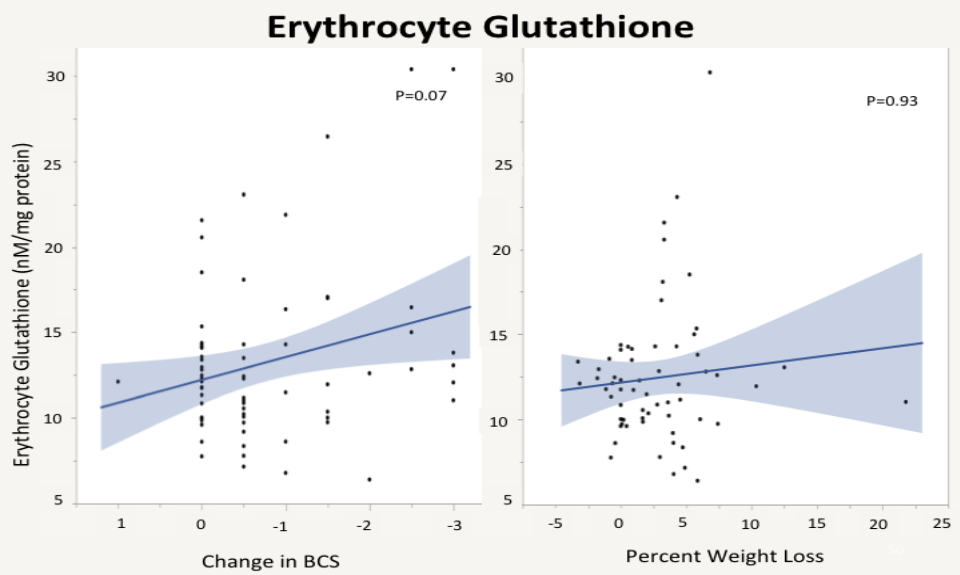

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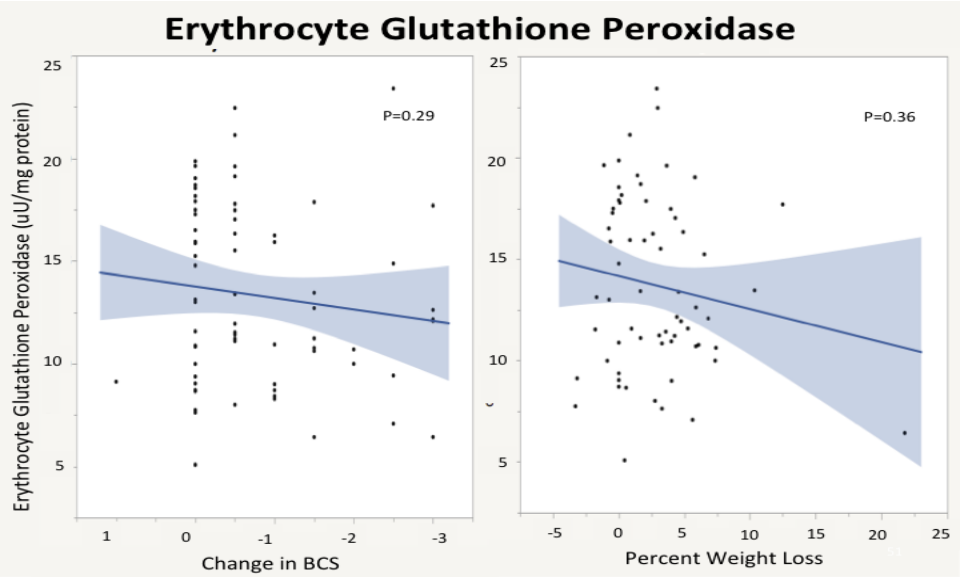

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Figure 2A-D: Mitochondrial Subpopulation Electron Transport Chain Enzyme Activities in Response to Change in Body Condition Score or Percent Weight Loss. Horses (n = 8) were enrolled in a baseline-sampling period lasting from d0 to d14, followed by a caloric restriction from d15 to d98, where from d15 to d43 horses received a 30% digestible energy reduction, and from d44 to d 98 horses received a 30% restriction from maintenance digestible energy. On d 0, 14, 28, 42, 70, and 98, middle gluteal muscle biopsies were taken and mitochondrial subpopulations isolated through differential centrifugation. Complex activities were measured spectrophotometrically depending on substrate production from each complex. The main effect of change in body condition score (BCS) and percent weight loss were analyzed for each variable, with data points representing values at corresponding points of BCS change or weight loss, respectively. Shading indicates confidence intervals.

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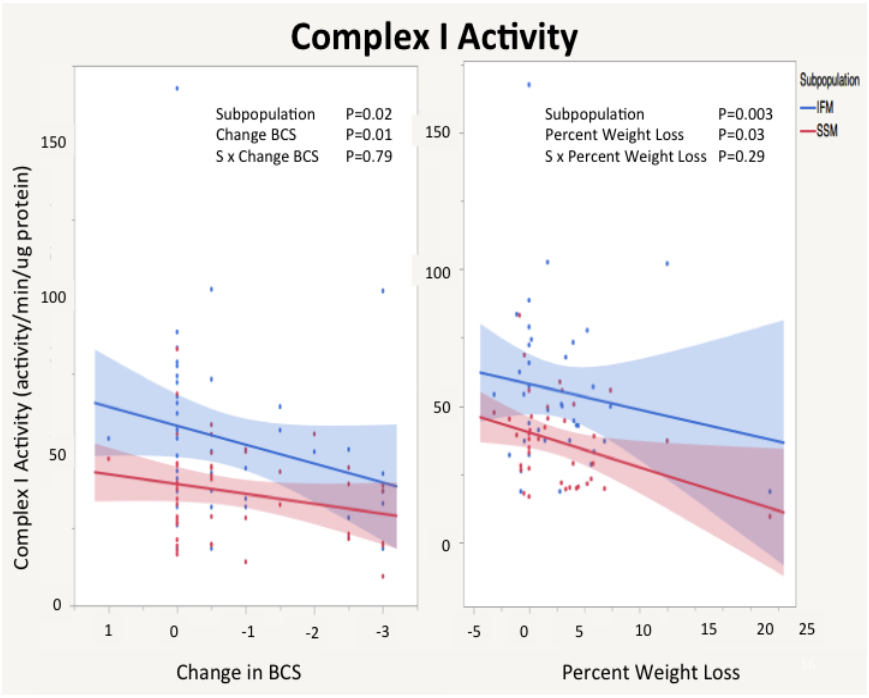

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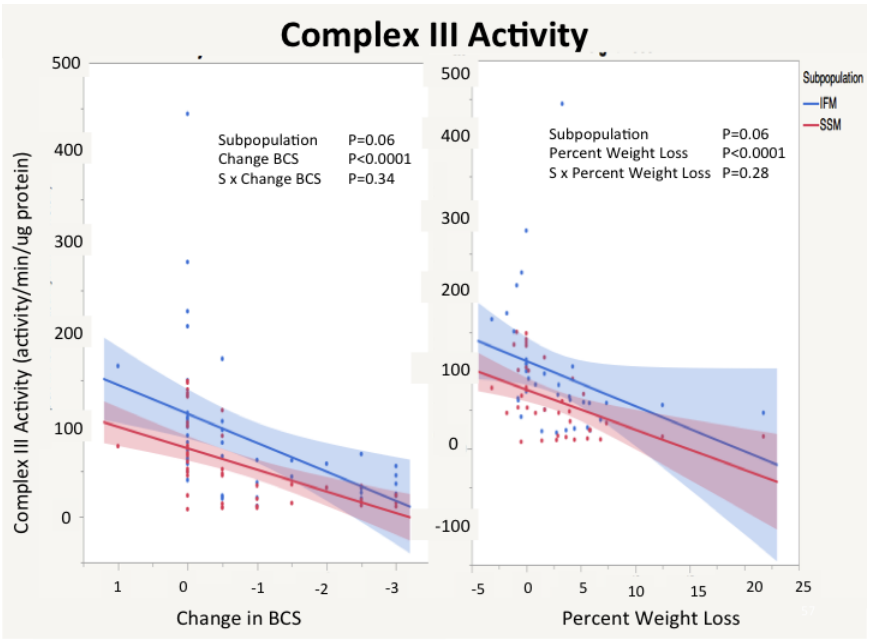

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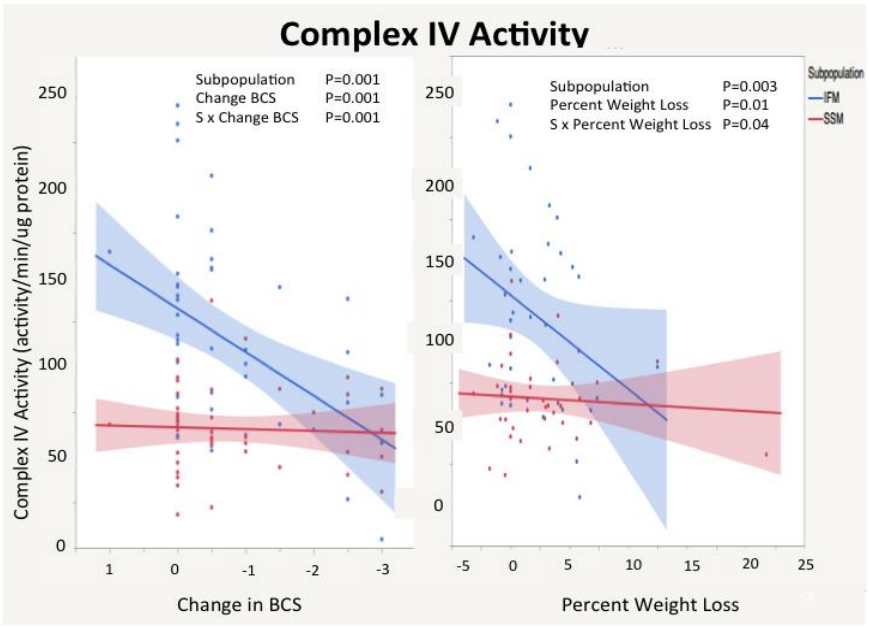

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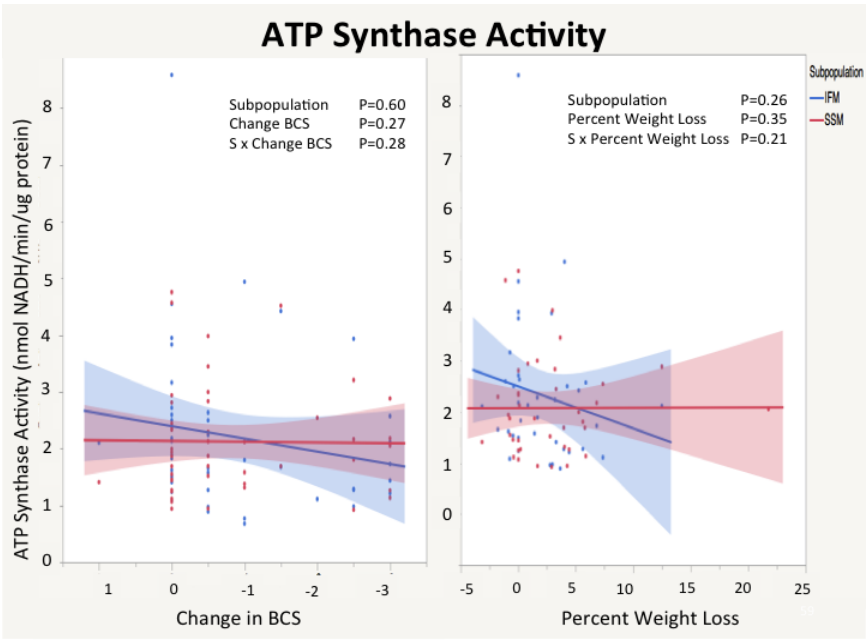

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Figure 3: Mitochondrial subpopulation citrate synthase activity as an indicator of population size in response to change in body condition score or percent weight loss. Mitochondrial Subpopulation Electron Transport Chain Enzyme Activities in Response to Change in Body Condition Score or Percent Weight Loss. Horses (n = 8) were enrolled in a baseline-sampling period lasting from d0 to d14, followed by a caloric restriction from d15 to d98, where from d15 to d43 horses received a 30% digestible energy reduction, and from d44 to d 98 horses received a 30% restriction from maintenance digestible energy. On d 0, 14, 28, 42, 70, and 98, middle gluteal muscle biopsies were taken and mitochondrial subpopulations isolated through differential centrifugation. Citrate synthase activity was measured spectrophotometrically. The main effect of change in body condition score (BCS) and percent weight loss were analyzed for each variable, with data points representing values at corresponding points of BCS change or weight loss, respectively. Shading indicates confidence intervals.

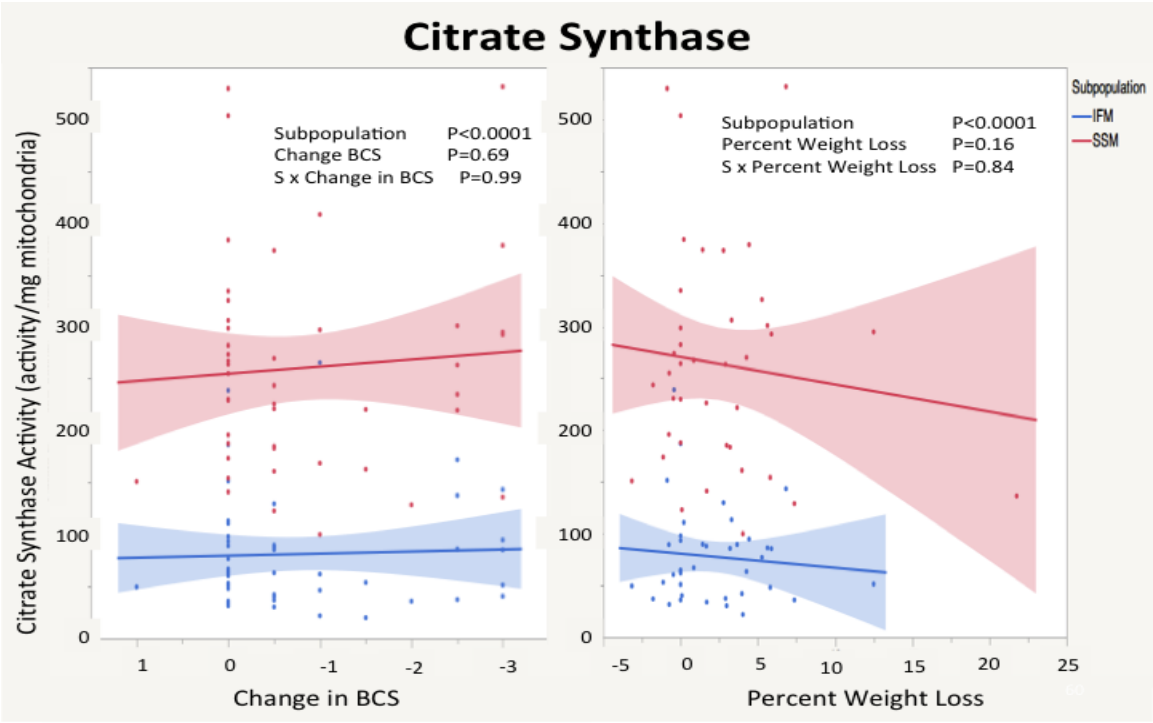

Figure 4: Lipid peroxidation in mitochondrial subpopulations in response to change in body condition score or percent weight loss. Horses (n = 8) were enrolled in a baseline-sampling period lasting from d0 to d14, followed by a caloric restriction from d15 to d98, where from d15 to d43 horses received a 30% digestible energy reduction, and from d44 to d 98 horses received a 30% restriction from maintenance digestible energy. On d 0, 14, 28, 42, 70, and 98, middle gluteal muscle biopsies were taken and mitochondrial subpopulations isolated through differential centrifugation. Mitochondrial lipid peroxidation was measured spectrophotometrically, and was normalized to total lipid content determined via a sulfo-phosphovanillin assay. The main effect of change in body condition score (BCS) and percent weight loss were analyzed for each variable, with data points representing values at corresponding points of BCS change or weight loss, respectively. Shading indicates confidence intervals.

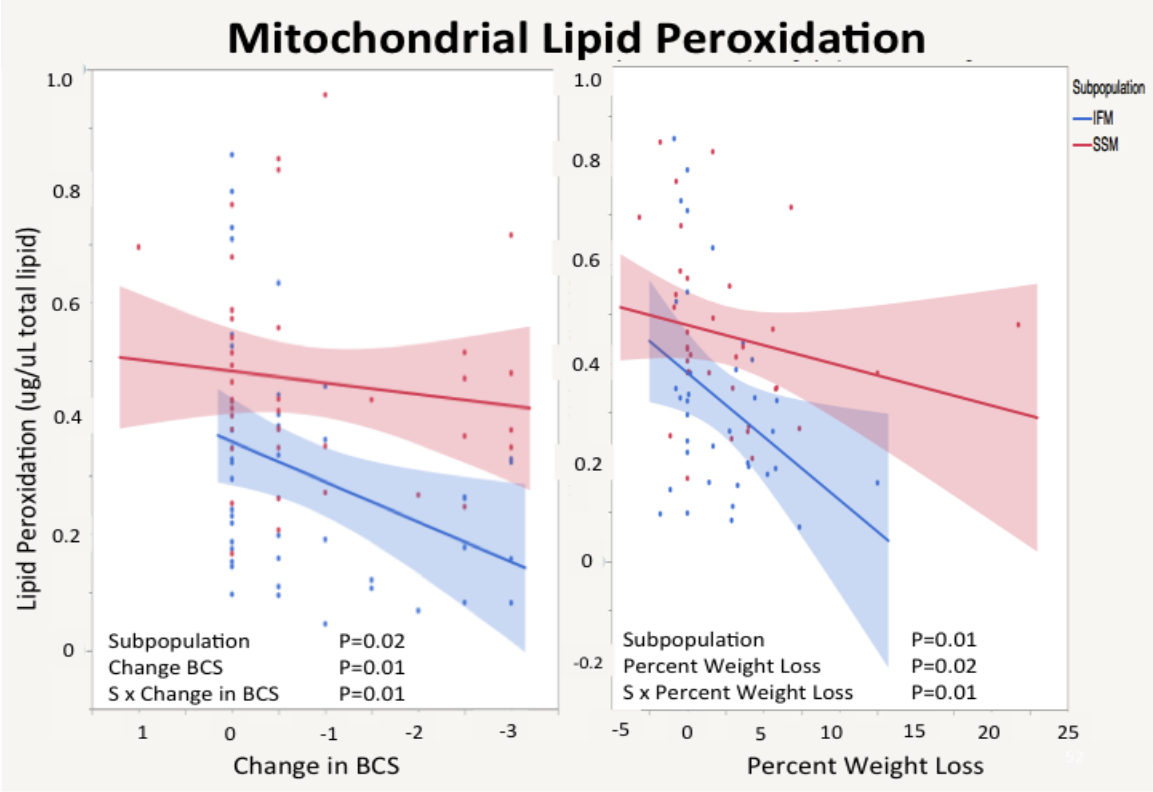

Table 5: Correlation of Morphometric Measurements, Complex Activity and Circulating Oxidant Status Markers[1]

	Cresty Neck Score		Nitric Oxide		Glutathione		Citrate Synthase SSM		Lipid Peroxidation SSM		Lipid Peroxidation IFM	
	R	P value	R	P value	R	P value	R	P value	R	P value	R	P value
Rump Fat Thickness	0.63	<0.0001	0.16	0.26	-0.41	0.004	-0.12	0.43	0.01	0.94	0.37	0.01
Percent Fat	0.63	<0.0001	0.15	0.31	-0.42	0.003	-0.13	0.41	0.03	0.88	0.38	0.01
Complex I - SSM	-0.09	0.53	0.33	0.02	-0.25	0.09	0.12	0.42	0.17	0.29	0.37	0.01
Complex III - SSM	0.46	0.002	0.06	0.69	-0.27	0.06	0.07	0.65	-0.04	0.8	0.40	0.01
Complex IV - SSM	-0.04	0.77	0.09	0.53	-0.16	0.29	-0.43	0.002	0.33	0.04	0.05	0.74
Complex IV - IFM	0.43	0.003	0.06	0.72	-0.05	0.75	-0.16	0.3	-0.33	0.04	0.06	0.69
Citrate Synthase - IFM	0.04	0.77	0.24	0.09	0.19	0.19	0.61	<0.0001	-0.14	0.41	0.57	<0.0001
Lipid Peroxidation - IFM	0.35	0.02	0.46	0.002	-0.22	0.14	0.51	0.0004	0.33	0.04		

[1]P values reflect significance and trends identified with bonferroni corrections, where significance determined at $p \leq 0.0021$ and trends noted when $p \leq 0.0052$.

CHAPTER IV: LIMITATIONS

As with any research project, limitations are inherently present and must be considered when interpreting results and looking forward to future research ideas. Specifically with regard to this project, limitations included sample size, time of sampling, species specific challenges, location of the project relative to analysis, and body condition of the subjects.

Inclusion criteria for this study resulted in a relatively small sample size relative to initial expected subject number. Upon analysis of results, specifically from both mitochondrial complex activities and minimal model output, large individual variation was observed. While consideration was taken to account for variability caused by gender, age, date and subpopulation where appropriate, additional comparisons may have been made had there been a larger total sampling population. For example, in the current study horses displayed a wide range of percent weight loss within observed BCS; had a larger sample size been possible, a natural break between “high” and “low” percentages of weight loss may have been revealed. The possibility remains that information regarding metabolic alterations with regard to weight loss is masked behind large variation. Increasing the sample size and allocating for natural breaks within the population could lend explanation to the variation observed in this study and alter overall interpretation of changes associated with obesity and subsequent weight loss.

Labor limitations and available stall space were also of concern during this study. All possible means to ensure horses were fed, managed and sampled in the same manner were taken, yet it was not fesable to collect fasting blood samples for analysis of

circulating metabolic indicators. Since samples were collected post-prandially, results were inconclusive. As the baseline FSIGT samples were collected after an overnight fast, we elected to evaluate these samples for circulating NEFA and TG levels (insulin and glucose concentrations measured for minimal model analysis). Unfortunately due to the timing of each FSIGT, this only provides for beginning and end-point information on these markers, leaving out samples from the weight loss period. Looking forward, management of horses so that fasting samples can be collected should be discussed for feasibility prior to the start of the research project. Additionally, inclusion of other markers that are not affected by fasting or fed states, including hemoglobin A1C or other glycosylated end products may also be of interest if fasting samples cannot be collected.

Unfortunately, while working with livestock species many analysis procedures do not transfer easily. For example, while hemoglobin A1C is considered a valid method for analyzing advancement of hyperglycemia in humans and rodents, initial attempts at transferring procedures to the horse have produced variable data. Additionally with regard to this study, measurement of specific oxidant status markers such as hydrogen peroxidase, manganese and copper/zinc superoxide dismutase have proven difficult to analyze with repeatable results. Analysis of genetic messages has also proven difficult, as the equine genome is not well annotated and many gene sequences are either unavailable or predicted. Thus, the measurements reported in this manuscript reflect assays previously validated in the horse. Further work is needed to accurately transition the aforementioned and other methodologies for analysis on equine samples.

In addition to the species-specific confines present in this study, travel limitations placed further restrictions on sample analysis procedures. As all samples were collected in Murfreesboro, TN and analysis was completed in Morgantown, WV samples were frozen at either -20 or -80 ° C until transport and measurement was completed. Many measurements to analyze mitochondrial number, size, respiration and functionality must be completed on fresh, not frozen, isolations. Moreover, analysis of complex II activity requires fresh mitochondria and therefore was not feasibly possible during this study. Looking forward, consideration should be given to alternate methods of sampling or locations in order to fully analyze mitochondrial parameters.

As previously mentioned, horses were body condition scored prior to entry into the study. While we did account for overall obesity by using this method, hindsight suggests we should have also taken CNS into account. The horses on this project had CNS ranging between 2.5 to 3 during the baseline sampling period, which is considered a moderate score. Selecting horses with a CNS score of 3.5 or greater, it is possible we would have observed different results as horses would have been more obese. Further, as amount of neck crest fat can signify a transition into an unhealthy metabolic state (Carter et al., 2009), selecting horses with higher CNS could provide insight into the transition from "metabolically healthy obese" to "metabolically unhealthy". Degree of adiposity in the neck crest region should be considered when designing future studies, especially if metabolic dysfunction is to be evaluated.

CHAPTER IV: OVERALL IMPLICATIONS, CONCLUSIONS AND FUTURE DIRECTIONS

Obesity in the equine industry has continued to increase over time, without predisposition for breed, discipline or region. Viable information regarding the progression of metabolic changes associated with equine obesity and subsequent weight loss are critical to proper management and understanding of species-specific metabolic processes. Knowledge gained from this study has the capacity to influence not only nutritional interventions but also catalyze the industry's interpretation of equine performance and evolutionary advantages.

Evaluation of circulating biomarkers suggests horses were beginning to show signs of metabolic alterations as signified by the elevated nitrate and reduction in reduced glutathione concentrations. Combined with the lack of changes seen in most minimal model parameters, it is hypothesized that horses were metabolically healthy despite their obesity. Although cellular dysfunction is typically reflected in circulating markers, it is plausible that obesity-related metabolic changes were in the primary stages of development. Insulin sensitivity displayed improvements with weight loss, signifying some alteration in signaling or sensitivity at the tissue level, yet AIRg was not affected. Had a more advanced stage of obesity been allowed to develop, it is suspected that more advanced dysfunction would have developed. This "metabolically healthy obese" phenotype may be very delicate, easily transforming into a disease state, yet providing some insight as to compensatory mechanisms behind the commonly accepted dysfunctions associated with excessive adipose deposition.

Equine mitochondria is surprisingly unstudied, especially due to the competitive athletic nature of the equine industry. Based on the results of this study, it is evident that a species-related difference in coping mechanisms are present in the horse when directly compared to the human or rodent models. It is commonly regarded that horses provide an excellent model for human exercise performance and to a certain extent this theory remains true. While horses display a similar thermoregulatory system to that of a human, it is well known that horses possess numerous physiological adaptations contributing to their superior athletic ability. Without excessive speculation, it is feasible that the mitochondria also play a significant role in the performance of the equine athlete. Respiration levels of SSM and IFM from equine middle gluteal muscle compared to gastrocnemius and soleus mitochondrial subpopulation isolations C57/Black 6 displayed similar activity levels, yet substrate utilization time was much faster for both equine populations (Zambito et al., 2013).

When combined with the data shown from this project, it is even more evident that horses display unique physiological mechanisms with respect to mitochondrial function. As horses are known for their ability to naturally blood dope via splenic contraction, the mitochondria may possess the ability to better handle large amounts of oxygen delivery without subsequent oxidative damage. This inherent protection may allow for equine mitochondria to possess more malleable characteristics to tolerate altering physiological environments. Return to a nearly equal level of complex activity between SSM and IFM displayed in this data could signify a more equal role between mitochondrial

subpopulations in a healthy state; a theory vastly differing from the suggested roles of SSM and IFM in humans and rodents alike (Kelley et al., 2002b; Menkishova et al., 2006; Ritov et al., 2005; Toledo et al., 2007). Unfortunately, due to lack of research on this specific topic, many of the proposed conclusions are based off negative comparisons with data in other species and not from completed works within the equine population. Mitochondrial research has an incredible depth of untapped potential due to the application of the knowledge base to athletic performance, cloning and reproduction and general animal health. Much more work is needed in this area to fill in the missing pieces as to the unique qualities of equine mitochondria.

Overall, it is evident that differences within the obese horse population exist between those considered metabolically healthy and those displaying signs of severe metabolic impairment. Excessive fat deposition in specific regions, including the neck crest and abdomen, may require more attention when determining how to manage and care for obese horses. From a scientific standpoint, the central dogma surrounding overweight horses and the incidence of metabolic dysfunction may need to be re-evaluated with regard to physiological factors and onset of disease states.

Looking forward, much work is needed with regard to mitochondrial function and obesity related metabolic changes in the horse. Classification of healthy equine mitochondria is necessary to understand not only the role of mitochondria on athletic performance but also propagation of disease states. Based on research in other species it remains plausible that understanding of many equine-related diseases can lie in evaluation of mitochondrial

function. Also, as regional fat deposition appears to play a role in obesity-related metabolic dysfunction research is needed to determine rates of deposition in different areas and their relationship to overall metabolism. Lastly, an in depth evaluation of the presence of a “metabolically healthy obese” phenotype is needed to classify how the industry manages obese horses. By understanding the physiology behind obesity related changes in glucose and lipid metabolism can drastically alter how we view the obese/overweight horse in both the performance and recreational setting.

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TEACHING EXPERIENCE

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AGBI 410 Introduction to Biochemistry: Teaching Assistant

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A&VS 281 Equine Management and Training: Teaching Assistant
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A&VS 293V Veterinary Anatomy Lab: Teaching Assistant

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A&VS 293W Equestrian Technologies: Instructor
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RESEARCH PUBLICATIONS AND PRESENTATIONS

Changes in Oxidant Status in Response to Weight Loss in Mature Light-Type Horses
EH Hoblitzell, KM Barnes, JL Zambito, HS Spooner
Submitted to 2014 Midwest ASDS ASAS Meeting

Effects of Weight Loss on Lipolysis and Skeletal Muscle Genetic Markers of Beta-Oxidation
M Bush, KM Barnes, JL Zambito, HS Spooner
Submitted to the Experimental Biology 2014 Meeting

Weight Loss Influence on Glucose Tolerance and Metabolic Markers in the Mature Horse
JL Zambito, HS Spooner, RM Hoffman, KM Barnes
Submitted to the Experimental Biology 2014 Meeting

Influence of Weight Loss on Mitochondrial Function in the Mature Horse
JL Zambito, CE Nichols, KM Barnes, HS Spooner, JM Hollander
Submitted to the International Conference on Equine Exercise Physiology 2015 Meeting

Effects of Resveratrol Supplementation on Glycemic Response and Oxidant Status in Exercising Aged Quarter Horse Geldings
JL Zambito, HS Spooner, RM Hoffman
Presented at the 2012 Joint Annual Meeting, Phoenix, AZ

Effects of Oral Resveratrol Supplementation on Insulin Sensitivity and Reproductive Cyclicity in Obese Mares on Pasture
KS Kohlhass, JL Zambito, HS Spooner, RC Boston, ML Rhodes, JW Knight, RK Splan

Journal of Equine Veterinary Science, 33:370-371, 2013

Comparison of Krill Oil and Fish Oil Supplementation on Serum and Tissue Fatty Acid Profiles in Horses
LE Bowen, HS Spooner, JL Zambito, KM Barnes
Journal of Equine Veterinary Science, 33: 342, 2013

Effects of Food Extracts on Plasma Electrolyte Concentration and Strong Ion Difference During Exercise and Recovery in Standardbred Mares
JL Zambito, KH McKeever, JM Streltsova, NR Liburt
Journal of Equine Veterinary Science, 29:479-480, 2009.
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The Effect of Age and Exercise Training on Insulin Sensitivity, Fat and Muscle Tissue Cytokine Profiles, and Body Composition of Old and Young Standardbred Mares
NR Liburt, MN Fugaro, K Malinowski, EK Wunderlich, JL Zambito, DW Horohov, A Betancourt, RC Boston, RJ Geor , J Onishi, KH McKeever
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APPENDIX A

Horse	Day	Plasma Nitrate (mg/dL)	Erythrocyte Glutathione (nMol/mg protein)	Erythrocyte Glutathione Peroxidase (uU/mg protein)	Serum NEFA (mg/mL)	Plasma TG (mg/mL)	Glucose (mg/dL)	Insulin (mU/L)
Boots	0	4.57	21.56	0.0076	0.483	12.22	108.69	2.80
Boots	14	7.52	14.09	0.0090	0.188	21.98	127.07	4.82
Boots	21	7.48	20.56	0.0108	0.192	34.22	126.09	4.45
Boots	28	11.87	18.51	0.0116	0.306	24.72	108.87	3.69
Boots	35	22.57	12.81	0.0152	0.124	38.44	133.17	5.27
Boots	42	16.17	15.34	0.0190	0.354	22.98	111.04	7.18
Boots	56	11.97	11.95	0.0135	0.114	17.46	127.33	2.38
Boots	70	24.89	26.44	0.0064	0.276	34.68	95.91	2.39
Boots	98	4.00	13.06	0.0177	0.240	10.32	114.81	2.68
Daphne	0	20.76	8.61	0.0175	0.075	18.48	113.65	12.70
Daphne	14	14.27	10.02	0.0109	0.074	46.32	91.63	6.16
Daphne	21	16.52	12.12	0.0159	0.297	20.84	104.55	4.62
Daphne	28	12.48	9.98	0.0182	0.157	18.1	112.52	5.04
Daphne	35	10.01	8.36	0.0119	0.451	149.32	122.64	4.47
Daphne	42	5.40	9.86	0.0187	0.131	18.4	107.54	6.55
Daphne	56	15.43	8.62	0.0109	0.114	31.46	143.55	3.31
Daphne	70	10.25	14.26	0.0083	0.094	21.7	134.84	7.27
Daphne	98	5.18	12.06	0.0121	0.239	35.94	133.27	9.22
Getda	0	18.33	13.56	0.0100	0.575	18.46	96.39	3.88
Getda	14	15.92	12.31	0.0087	0.080	22.42	118.05	17.82
Getda	21	21.84	14.27	0.0087	0.258	25.7	93.51	2.47
Getda	28	16.29	10.07	0.0134	0.316	26.34	151.36	1.84
Getda	35	8.39	14.29	0.0162	0.432	38.82	99.73	3.16
Getda	42	16.14	10.87	0.0080	0.413	22.92	121.79	3.15
Getda	56	49.56	17.00	0.0112	0.113	29.84	125.06	2.57
Getda	70	8.75	30.38	0.0094	0.248	31.22	194.11	2.23
Getda	98	10.33	30.38	0.0121	0.057	13.22	101.26	8.76
JR	0	6.08	12.11	0.0091	0.073	27.82	110.82	1.99
JR	14	6.21	11.76	0.0179	0.172	45.86	112.26	1.97
JR	21	3.74	11.73	0.0116	0.157	33.6	97.53	3.71
JR	28	4.66	7.79	0.0224	0.157	39.06	79.35	2.02
JR	35	4.41	7.16	0.0163	0.182	29.06	43.48	2.02
JR	42	4.98	6.79	0.0090	0.265	26.68	108.36	2.27
JR	56	16.15	6.40	0.0107	0.073	17.34	99.85	2.31
JR	70	9.70	12.60	0.0100	0.280	20.7	112.26	2.04

Horse	Day	Plasma Nitrate (mg/dL)	Erythrocyte Glutathione (nMol/mg protein)	Erythrocyte Glutathione Peroxidase (uU/mg protein)	Serum NEFA (mg/ mL)	Plasma TG (mg/ mL)	Glucose (mg/dL)	Insulin (mU/L)
Pistol	0	8.56	12.42	0.0115	0.176	8.04	97.36	4.09
Pistol	14	10.59	10.85	0.0148	0.064	15.22	161.46	7.09
Pistol	21	13.91	13.39	0.0077	0.144	20.22	108.73	3.52
Pistol	28	7.53	11.78	0.0196	0.072	16.72	103.97	4.46
Pistol	35	18.12	13.49	0.0211	0.179	23.18	100.17	2.77
Pistol	42	6.53	12.47	0.0173	0.170	28.82	108.79	5.20
Pistol	56	6.71	11.48	0.0159	0.068	19.68	128.33	2.98
Pistol	70	8.21	21.88	0.0087	0.253	30.82	126.45	6.11
Pistol	98	11.28	12.84	0.0234	0.158	15.96	145.46	8.29
Romeo	0	20.57	14.14	0.0159	0.184	26.06	113.14	6.99
Romeo	14	11.83	14.37	0.0199	0.073	23.72	152.51	11.42
Romeo	21	13.79	23.06	0.0170	0.291	31.96	116.67	4.12
Romeo	28	19.02	18.08	0.0155	0.318	30.96	74.13	2.23
Romeo	35	13.84	11.00	0.0114	0.389	35.56	114.11	2.74
Romeo	42	15.48	10.22	0.0196	0.198	27.2	117.78	3.35
Romeo	56	25.70	10.01	0.0108	0.223	33.58	148.08	2.61
Romeo	70	9.59	16.46	0.0149	0.154	38.04	136.13	1.62
Romeo	98	6.83	13.80	0.0126	0.116	22.84	151.83	3.22
Susie	0	9.88	7.76	0.0165	0.127	11.82	99.98	7.08
Susie	14	10.23	11.74	0.0185	0.044	17.58	115.04	8.46
Susie	21	18.26	12.95	0.0131	0.234	19.96	38.89	5.99
Susie	28	14.32	9.72	0.0178	0.163	22.96	106.75	4.06
Susie	35	13.94	10.55	0.0111	0.119	13.98	91.01	5.00
Susie	42	17.87	12.29	0.0191	0.206	10.3	107.77	5.31
Susie	56	14.33	10.36	0.0179	0.061	22.44	102.77	6.31
Susie	70	12.87	16.35	0.0084	.	25.84	129.14	5.86
Susie	98	5.25	15.00	0.0071	0.125	30.7	111.20	5.53
Sweetie	0	18.99	11.33	0.0130	0.149	29.18	104.77	9.02
Sweetie	14	6.91	9.61	0.0094	0.131	29.98	107.61	19.05
Sweetie	21	22.88	9.61	0.0051	0.255	30.82	139.30	7.06
Sweetie	28	6.98	14.29	0.0112	0.273	42.96	125.60	8.49
Sweetie	35	3.69	11.16	0.0134	0.106	22.72	96.55	7.51
Sweetie	42	6.21	9.20	0.0175	0.060	22.22	118.33	5.82
Sweetie	56	6.70	9.74	0.0106	0.107	26.1	95.12	6.53
Sweetie	70	11.40	17.07	0.0127	0.225	23.2	147.66	2.45
Sweetie	98	5.74	11.03	0.0064	0.228	22.72	131.99	3.37

1848

Appendix B

Horse	Horse Number	Age (yr)	Sex	Breed	Wither Height	Body Length	Neck Length
Boots	1	10	M	Quarter Horse	142.2	157.5	71.1
Daphne	2	6	M	Tennessee Walker	148.0	137.2	71.1
Getda	3	13	M	Standardbred	160.0	156.2	101.6
JR	4	19	G	Quarter Horse	149.9	149.9	86.4
Pistol	5	5	M	Quarter Horse	147.3	152.4	81.3
Romeo	6	10	G	Quarter Horse	170.8	165.1	96.5
Susie	7	18	M	Quarter Horse	151.8	158.8	95.3
Sweetie	8	15	M	Quarter Horse	157.5	165.1	104.1

1851

Horse	Day	Girth Circ. (cm)	Abd. Circ. (cm)	Neck Crest Height	Neck Circ. at 0.25 NL (cm)	Neck Circ. at 0.5 NL (cm)	Neck Circ. at 0.75 NL (cm)
Boots	0	187.96	208.28	10.16	77.47	95.25	110.49
Boots	14	189.23	200.66	10.16	78.40	88.90	109.22
Boots	28	187.50	201.00	10.00	73.50	89.00	104.00
Boots	42	184.00	195.00	10.00	73.00	89.00	105.00
Boots	70	182.00	190.00	8.00	74.50	90.00	106.00
Boots	98	179.00	187.00	7.50	70.00	90.00	103.00
Daphne	0	184.15	196.85	12.07	72.39	91.66	106.64
Daphne	14	189.00	198.00	12.00	76.00	90.00	105.00
Daphne	28	184.00	191.00	11.50	73.00	90.00	112.50
Daphne	42	189.00	189.00	11.50	72.00	91.00	103.00
Daphne	70	183.00	190.00	10.00	78.00	92.00	107.00
Daphne	98	183.00	188.00	9.00	71.00	85.00	101.00
Getda	0	201.93	226.06	11.43	79.38	102.24	118.75
Getda	14	202.00	217.00	12.00	77.50	101.40	117.00
Getda	28	206.50	219.50	.	76.00	92.00	107.50
Getda	42	202.00	216.50	9.25	76.00	87.50	105.00
Getda	70	220.50	221.50	8.50	74.00	93.50	104.00
Getda	98	.	.	7.00	72.00	91.50	102.00
JR	0	189.23	215.90	13.97	79.38	102.87	123.19
JR	14	187.00	213.00	15.24	78.00	91.00	113.00
JR	28	184.00	208.00	13.00	80.00	90.50	116.00
JR	42	182.00	286.00	10.00	78.00	91.00	110.00
JR	70	186.00	199.00	10.00	78.00	105.00	119.00
Pistol	0	186.69	208.28	10.80	69.85	91.44	109.22
Pistol	14	187.00	196.00	10.00	83.00	105.00	113.00
Pistol	28	189.00	203.00	9.00	68.50	93.00	107.00
Pistol	42	187.00	200.00	9.00	76.00	97.00	107.00
Pistol	70	179.50	182.50	10.00	73.50	85.50	106.00
Pistol	98	187.50	196.00	5.50	76.00	90.50	106.00
Romeo	0	212.09	231.14	12.07	87.00	106.68	125.73
Romeo	14	218.00	230.00	12.00	89.00	104.50	125.50
Romeo	28	210.00	233.00	11.00	89.00	104.00	128.00
Romeo	42	214.50	225.50	10.50	85.50	102.00	124.00
Romeo	70	208.00	228.00	9.00	82.00	98.00	127.00
Romeo	98	203.00	225.00	9.50	82.00	98.00	125.00

1852

Horse	Day	Girth Circ. (cm)	Abd. Circ. (cm)	Neck Crest Height	Neck Circ. at 0.25 NL (cm)	Neck Circ. at 0.5 NL (cm)	Neck Circ. at 0.75 NL (cm)
Susie	0	194.31	220.98	12.07	79.38	102.87	120.02
Susie	14	191.77	224.79	13.97	76.84	102.87	120.02
Susie	28	196.00	207.00	10.00	84.00	94.50	109.00
Susie	42	188.00	213.00	.	76.00	94.00	103.00
Susie	70	188.00	210.00	11.00	78.00	103.00	116.00
Susie	98	182.50	208.00	6.50	75.50	92.50	113.00
Sweetie	0	200.66	213.36	13.97	85.09	111.13	129.86
Sweetie	14	196.85	217.17	13.97	81.92	109.22	129.54
Sweetie	28	195.50	205.50	11.50	80.00	99.50	114.50
Sweetie	42	196.00	207.00	9.00	82.00	100.00	121.00
Sweetie	70	199.00	199.00	12.00	78.00	106.00	132.00
Sweetie	98	197.00	195.00	6.00	77.50	90.00	108.00

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APPENDIX C

Horse	Day	BCS	CNS	Rump Fat Thickness (mm)	Percent Fat	Fat Mass (kg)	Fat Free Mass (kg)	Weight (kg)
Boots	0	8	3	9	7.39	36.4	455.9	492.3
Boots	14	8	3	8	6.85	34.9	474.2	509.1
Boots	28	8	3	8	6.85	33.0	449.3	482.3
Boots	42	8	3	9	7.39	35.5	444.1	479.5
Boots	70	6.5	2.5	7.5	6.57	30.3	430.6	460.9
Boots	98	5	2	3	4.11	18.3	427.1	445.5
Daphne	0	8	3	19	12.86	55.6	376.7	432.3
Daphne	14	8	3	19	12.86	55.4	375.1	430.5
Daphne	28	8	3	18	12.32	52.9	376.6	429.5
Daphne	42	8	3	18	12.32	52.1	371.1	423.2
Daphne	70	7	2.5	7	6.30	29.3	435.3	464.5
Daphne	98	5	2.5	10	7.94	32.7	378.7	411.4
Getda	0	8	2.5	5	5.21	30.1	547.7	577.7
Getda	14	8	2.5	10	7.94	45.5	527.3	572.7
Getda	28	7.5	2.5	9	7.39	41.6	521.5	563.2
Getda	42	7.5	2	6	5.75	32.0	524.8	556.8
Getda	70	5.5	2	3	4.11	22.9	533.9	556.8
Getda	98	5	2	3	4.11	21.9	511.7	533.6
JR	0	8	2.5	10	7.94	42.4	492.1	534.5
JR	14	7	2.5	8	6.85	35.5	482.7	518.2
JR	28	6.5	2	11	8.49	42.7	460.1	502.7
JR	42	6	2	8	6.85	34.0	463.2	497.3
JR	70	5	2	4	4.66	22.4	457.6	480.0
Pistol	0	7	2.5	9.5	7.67	37.8	455.0	492.7
Pistol	14	7.5	2.5	10	7.94	38.4	445.7	484.1
Pistol	28	7.5	.	11	8.49	41.5	448.0	489.5
Pistol	42	7.5	2.5	8	8.49	41.3	445.1	486.4
Pistol	70	6.5	2.5	12	9.03	37.6	378.3	415.9
Pistol	98	5	2	6.5	6.03	28.3	441.7	470.0
Romeo	0	8	2.5	12	9.03	62.3	627.3	689.5
Romeo	14	8	2.5	14	10.13	70.4	625.0	695.5
Romeo	28	7.5	2.5	8	6.85	46.1	627.1	673.2
Romeo	42	7.5	2.5	9	7.39	49.5	620.5	670.0
Romeo	70	5.5	2	9	7.39	45.5	570.0	615.5
Romeo	98	5	2	6.5	6.03	39.4	615.1	654.5

Horse	Day	BCS	CNS	Rump Fat Thickness (mm)	Percent Fat	Fat Mass (kg)	Fat Free Mass (kg)	Weight (kg)
Susie	0	7.5	2.5	10	7.94	43.3	502.1	545.5
Susie	14	7.5	2.5	10	7.94	43.0	498.4	541.4
Susie	28	7	2.5	9	7.39	40.0	500.9	540.9
Susie	42	7	2.5	8.5	7.12	38.0	495.6	533.6
Susie	70	6.5	2	8	6.85	36.1	490.8	526.8
Susie	98	5	2	10	7.94	40.6	470.3	510.9
Sweetie	0	8	3	14	10.13	63.1	559.7	622.7
Sweetie	14	8	2.5	11	8.49	52.5	565.7	618.2
Sweetie	28	7.5	3	9	7.39	43.8	548.1	591.8
Sweetie	42	7.5	3	9.5	7.67	45.5	548.1	593.6
Sweetie	70	6.5	2	6	5.75	32.1	525.6	557.7
Sweetie	98	5	2	4	4.66	22.5	461.1	483.6

1857

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APPENDIX D

Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)	Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)
Boots	1	-30	141.68	4.33	Boots	2	3	294.16	15.91
Boots	1	0	235.75	14.66	Boots	2	4	310.73	12.75
Boots	1	1	383.04	.	Boots	2	5	318.94	14.87
Boots	1	2	316.40	15.15	Boots	2	6	317.84	12.64
Boots	1	3	341.84	11.16	Boots	2	7	314.74	15.10
Boots	1	4	313.55	.	Boots	2	8	299.42	12.43
Boots	1	5	330.58	19.25	Boots	2	9	290.32	10.53
Boots	1	6	350.10	16.97	Boots	2	10	287.95	11.61
Boots	1	7	314.74	.	Boots	2	12	320.33	11.18
Boots	1	8	324.18	13.86	Boots	2	14	304.45	11.19
Boots	1	10	321.58	16.83	Boots	2	16	295.61	9.36
Boots	1	12	279.38	17.67	Boots	2	19	281.13	8.54
Boots	1	14	296.33	13.19	Boots	2	22	241.12	212.42
Boots	1	16	321.90	14.82	Boots	2	23	255.45	157.48
Boots	1	19	292.43	14.01	Boots	2	24	265.69	135.75
Boots	1	22	318.97	12.20	Boots	2	25	265.34	111.98
Boots	1	23	300.36	216.46	Boots	2	27	241.13	77.04
Boots	1	24	278.48	209.35	Boots	2	30	229.18	64.57
Boots	1	25	312.03	195.40	Boots	2	35	239.62	30.20
Boots	1	27	270.37	122.02	Boots	2	40	179.81	21.09
Boots	1	30	303.59	116.60	Boots	2	50	176.35	8.72
Boots	1	35	287.61	70.45	Boots	2	60	121.16	5.01
Boots	1	40	268.40	.	Boots	2	70	125.42	3.50
Boots	1	50	276.99	.	Boots	2	80	110.32	5.55
Boots	1	60	264.61	.	Boots	2	90	108.76	3.90
Boots	1	70	234.09	.	Boots	2	100	129.76	.
Boots	1	80	225.45	.	Boots	2	120	101.34	2.78
Boots	1	90	244.76	.	Boots	2	150	109.89	2.43
Boots	1	100	313.21	14.94	Boots	2	180	103.40	2.60
Boots	1	120	217.32	15.68	Daphne	1	-30	104.14	3.71
Boots	1	150	195.43	11.31	Daphne	1	0	275.64	3.10
Boots	1	180	161.08	5.26	Daphne	1	1	299.76	26.49
Boots	1	240	.	5.17	Daphne	1	2	282.86	28.15
Boots	2	-30	159.89	4.63	Daphne	1	3	314.47	26.30
Boots	2	0	136.29	3.90	Daphne	1	4	292.44	30.51
Boots	2	1	318.75	63.06	Daphne	1	5	323.67	29.17
Boots	2	2	315.23	13.18	Daphne	1	6	317.14	30.73

Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)	Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)
Daphne	1	7	305.82	31.35	Daphne	2	19	320.66	13.43
Daphne	1	8	328.55	35.77	Daphne	2	22	234.75	319.58
Daphne	1	10	289.76	36.57	Daphne	2	23	245.05	202.54
Daphne	1	12	266.90	.	Daphne	2	24	277.02	162.85
Daphne	1	14	326.60	29.17	Daphne	2	25	282.56	145.21
Daphne	1	16	319.96	26.91	Daphne	2	27	268.37	109.06
Daphne	1	19	279.35	27.65	Daphne	2	30	270.67	80.82
Daphne	1	22	323.11	319.06	Daphne	2	35	272.30	51.44
Daphne	1	23	323.41	276.25	Daphne	2	40	222.53	36.17
Daphne	1	24	270.46	235.25	Daphne	2	50	186.83	25.42
Daphne	1	25	312.21	205.59	Daphne	2	60	156.42	23.47
Daphne	1	27	304.55	175.44	Daphne	2	70	148.35	17.48
Daphne	1	30	303.59	143.07	Daphne	2	80	127.03	14.31
Daphne	1	35	322.79	100.61	Daphne	2	90	116.93	12.19
Daphne	1	40	275.57	83.83	Daphne	2	100	121.23	10.98
Daphne	1	50	283.08	61.73	Daphne	2	120	111.28	8.60
Daphne	1	60	253.30	56.73	Daphne	2	150	97.54	7.12
Daphne	1	70	202.07	55.26	Daphne	2	180	85.73	5.26
Daphne	1	80	192.61	54.56	Getda	1	-30	138.60	5.33
Daphne	1	90	184.95	56.36	Getda	1	0	114.40	7.82
Daphne	1	100	211.14	52.16	Getda	1	1	344.47	43.95
Daphne	1	120	154.28	37.99	Getda	1	2	339.88	49.32
Daphne	1	150	103.36	19.97	Getda	1	3	285.15	56.80
Daphne	1	180	79.34	10.27	Getda	1	4	291.63	58.64
Daphne	1	240	78.57	2.54	Getda	1	5	344.35	56.79
Daphne	2	-30	117.99	6.75	Getda	1	6	325.73	50.64
Daphne	2	0	110.45	5.80	Getda	1	7	307.23	50.23
Daphne	2	1	311.43	18.45	Getda	1	8	290.62	55.36
Daphne	2	2	330.59	18.26	Getda	1	9	.	.
Daphne	2	3	301.42	16.84	Getda	1	10	289.01	53.33
Daphne	2	4	340.42	19.77	Getda	1	12	299.19	42.29
Daphne	2	5	302.35	22.05	Getda	1	14	264.01	45.03
Daphne	2	6	299.04	.	Getda	1	16	260.86	42.39
Daphne	2	7	296.82	19.07	Getda	1	19	302.16	45.08
Daphne	2	8	308.08	17.79	Getda	1	22	266.90	495.47
Daphne	2	9	297.40	17.13	Getda	1	23	312.61	290.42
Daphne	2	10	296.06	14.90	Getda	1	24	283.87	297.12
Daphne	2	12	241.63	16.00	Getda	1	25	303.60	226.22

Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)	Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)
Daphne	2	14	306.41	13.30	Getda	1	27	292.28	189.35
Daphne	2	16	303.61	14.74	Getda	1	30	257.78	160.45
Getda	1	35	275.64	107.56	Getda	2	90	144.64	34.96
Getda	1	40	258.68	95.56	Getda	2	100	.	31.25
Getda	1	50	289.89	71.01	Getda	2	120	174.23	24.20
Getda	1	60	273.22	51.50	Getda	2	150	105.65	14.75
Getda	1	70	226.50	47.19	Getda	2	180	94.60	8.67
Getda	1	80	226.30	49.90	JR	1	-30	108.86	2.10
Getda	1	90	193.50	39.32	JR	1	0	105.99	2.69
Getda	1	100	184.48	40.32	JR	1	1	297.73	14.39
Getda	1	120	209.05	30.25	JR	1	2	337.42	19.13
Getda	1	150	308.45	25.52	JR	1	3	302.18	16.34
Getda	1	180	149.21	16.49	JR	1	4	329.24	18.32
Getda	1	240	113.62	6.84	JR	1	5	303.17	25.48
Getda	2	-30	120.66	10.00	JR	1	6	302.69	27.34
Getda	2	0	107.47	6.27	JR	1	7	295.08	21.44
Getda	2	1	266.81	56.90	JR	1	8	189.98	26.11
Getda	2	2	298.70	59.08	JR	1	10	299.49	23.60
Getda	2	3	319.24	63.57	JR	1	12	304.89	22.14
Getda	2	4	325.87	64.15	JR	1	14	274.79	18.00
Getda	2	5	267.30	60.61	JR	1	16	304.23	16.38
Getda	2	6	318.81	56.03	JR	1	19	313.16	14.86
Getda	2	7	298.33	45.81	JR	1	22	309.72	13.67
Getda	2	8	322.77	50.47	JR	1	23	295.35	302.64
Getda	2	9	330.95	50.70	JR	1	24	291.86	247.29
Getda	2	10	250.40	44.25	JR	1	25	288.14	173.85
Getda	2	12	314.18	35.23	JR	1	27	259.12	155.52
Getda	2	14	285.19	37.10	JR	1	30	230.51	121.99
Getda	2	16	277.54	33.94	JR	1	35	261.32	91.19
Getda	2	19	302.05	29.91	JR	1	40	237.79	53.21
Getda	2	22	270.00	358.34	JR	1	50	324.91	34.54
Getda	2	23	266.52	249.23	JR	1	60	168.11	22.71
Getda	2	24	262.68	142.11	JR	1	70	202.77	18.25
Getda	2	25	257.66	173.82	JR	1	80	167.08	17.39
Getda	2	27	264.22	142.79	JR	1	90	146.75	14.60
Getda	2	30	233.61	129.90	JR	1	100	157.64	10.97
Getda	2	35	271.35	69.62	JR	1	120	136.10	9.93
Getda	2	40	248.74	54.66	JR	1	150	124.94	9.18

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Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)	Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)
Getda	2	50	232.23	43.40	JR	1	180	100.26	4.87
Getda	2	60	240.93	37.20	JR	1	240	287.76	4.46
Getda	2	70	177.26	41.98	JR	1	240	78.04	1.71
Getda	2	80	174.74	18.40	JR	2	-30	148.64	2.42
JR	2	0	130.27	2.50	Pistol	1	7	342.84	10.41
JR	2	1	320.24	.	Pistol	1	8	313.05	.
JR	2	2	327.41	6.37	Pistol	1	10	289.89	11.97
JR	2	3	315.76	8.58	Pistol	1	12	314.55	7.89
JR	2	4	290.79	8.02	Pistol	1	14	290.28	9.14
JR	2	5	285.37	25.93	Pistol	1	16	264.40	9.08
JR	2	6	311.66	9.88	Pistol	1	19	299.17	8.19
JR	2	7	311.00	9.23	Pistol	1	22	287.48	8.63
JR	2	8	315.39	14.32	Pistol	1	23	281.68	395.19
JR	2	9	269.32	7.45	Pistol	1	24	242.53	252.88
JR	2	10	272.96	7.36	Pistol	1	25	268.86	192.34
JR	2	12	336.17	5.89	Pistol	1	27	317.90	163.09
JR	2	14	289.88	6.07	Pistol	1	30	237.00	119.10
JR	2	16	291.67	6.45	Pistol	1	35	224.42	353.29
JR	2	19	282.97	6.71	Pistol	1	40	183.77	59.27
JR	2	22	289.91	321.57	Pistol	1	50	176.49	29.60
JR	2	23	322.04	198.99	Pistol	1	60	155.34	21.75
JR	2	24	262.25	176.21	Pistol	1	70	127.26	11.86
JR	2	25	288.42	158.79	Pistol	1	80	113.37	7.75
JR	2	27	252.68	217.74	Pistol	1	90	125.19	7.90
JR	2	30	250.13	120.06	Pistol	1	100	126.05	5.29
JR	2	35	220.81	37.16	Pistol	1	120	85.34	5.33
JR	2	40	214.27	20.77	Pistol	1	150	65.95	3.71
JR	2	50	184.37	50.87	Pistol	1	180	98.39	1.84
JR	2	60	144.35	9.03	Pistol	1	240	96.58	2.98
JR	2	70	119.47	119.93	Pistol	2	-30	143.88	4.21
JR	2	80	114.55	13.51	Pistol	2	0	124.62	4.40
JR	2	90	103.97	3.95	Pistol	2	1	337.21	13.71
JR	2	100	117.25	7.12	Pistol	2	2	336.26	12.13
JR	2	120	97.26	3.08	Pistol	2	3	332.70	.
JR	2	150	87.47	2.31	Pistol	2	4	329.72	11.67
JR	2	180	86.44	2.96	Pistol	2	5	330.89	12.30
Pistol	1	-30	111.13	4.13	Pistol	2	6	334.39	9.36
Pistol	1	0	299.54	2.27	Pistol	2	7	304.52	8.44

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Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)	Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)
Pistol	1	1	331.75	10.63	Pistol	2	8	291.72	11.81
Pistol	1	2	286.25	10.49	Pistol	2	9	341.71	11.48
Pistol	1	3	330.15	8.69	Pistol	2	10	318.60	11.85
Pistol	1	4	296.46	9.64	Pistol	2	12	278.93	.
Pistol	1	5	285.39	9.58	Pistol	2	14	278.68	12.85
Pistol	1	6	292.23	11.08	Pistol	2	16	268.34	10.90
Pistol	2	19	286.03	9.00	Romeo	1	50	282.39	55.03
Pistol	2	22	267.53	361.97	Romeo	1	60	235.25	53.85
Pistol	2	23	297.68	107.58	Romeo	1	70	202.24	51.10
Pistol	2	24	276.09	131.03	Romeo	1	80	192.67	44.16
Pistol	2	25	275.05	108.16	Romeo	1	90	186.06	37.31
Pistol	2	30	225.25	65.84	Romeo	1	100	157.54	28.18
Pistol	2	35	168.86	35.75	Romeo	1	120	211.14	31.38
Pistol	2	40	140.53	20.30	Romeo	1	150	337.94	14.72
Pistol	2	50	86.59	9.27	Romeo	1	180	255.43	9.05
Pistol	2	60	69.00	6.77	Romeo	1	240	105.64	2.51
Pistol	2	70	68.62	6.57	Romeo	2	-30	140.70	5.13
Pistol	2	80	57.62	4.32	Romeo	2	0	129.23	4.53
Pistol	2	90	52.74	3.66	Romeo	2	1	281.98	14.31
Pistol	2	100	50.60	2.63	Romeo	2	2	322.27	15.36
Pistol	2	120	68.77	1.82	Romeo	2	3	274.44	16.36
Pistol	2	150	87.14	1.60	Romeo	2	4	246.25	14.74
Pistol	2	180	102.43	2.04	Romeo	2	5	302.45	15.45
Romeo	1	-30	114.31	5.19	Romeo	2	7	287.79	17.11
Romeo	1	0	117.09	3.70	Romeo	2	8	336.73	15.84
Romeo	1	1	348.64	17.50	Romeo	2	9	314.70	18.54
Romeo	1	2	332.08	25.20	Romeo	2	10	307.11	19.37
Romeo	1	3	346.00	23.85	Romeo	2	12	316.01	18.87
Romeo	1	4	367.21	27.98	Romeo	2	14	302.65	15.43
Romeo	1	5	311.30	29.71	Romeo	2	16	314.29	17.40
Romeo	1	6	327.86	27.57	Romeo	2	19	320.92	18.92
Romeo	1	7	326.15	24.94	Romeo	2	22	310.95	.
Romeo	1	8	302.74	34.96	Romeo	2	23	243.62	186.49
Romeo	1	10	292.95	25.51	Romeo	2	24	357.99	176.86
Romeo	1	12	113.74	23.63	Romeo	2	25	308.35	147.98
Romeo	1	14	320.17	20.59	Romeo	2	27	323.50	124.21
Romeo	1	16	295.35	26.92	Romeo	2	30	245.06	93.96
Romeo	1	19	117.29	21.39	Romeo	2	35	250.84	72.19

Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)	Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)
Romeo	1	22	292.27	522.57	Romeo	2	40	238.32	49.50
Romeo	1	23	287.28	324.23	Romeo	2	50	215.14	36.65
Romeo	1	24	321.94	257.02	Romeo	2	60	176.18	28.95
Romeo	1	25	288.66	227.44	Romeo	2	70	166.94	24.43
Romeo	1	27	257.60	160.16	Romeo	2	80	169.77	19.03
Romeo	1	30	266.31	125.96	Romeo	2	90	144.03	15.19
Romeo	1	35	247.16	88.73	Romeo	2	100	130.09	10.68
Romeo	1	40	241.42	72.41	Romeo	2	120	111.29	7.76
Romeo	2	150	132.52	5.81	Susie	2	3	330.13	47.42
Romeo	2	180	106.01	6.97	Susie	2	4	290.34	49.33
Susie	1	-30	151.02	4.70	Susie	2	5	256.96	54.56
Susie	1	0	134.90	3.37	Susie	2	6	291.88	60.87
Susie	1	1	345.60	34.23	Susie	2	7	293.56	60.34
Susie	1	2	327.47	38.59	Susie	2	8	.	.
Susie	1	3	378.50	41.22	Susie	2	9	282.61	49.29
Susie	1	4	329.47	43.46	Susie	2	10	266.52	66.02
Susie	1	5	289.63	43.47	Susie	2	12	285.17	515.54
Susie	1	6	296.18	44.92	Susie	2	14	240.66	65.98
Susie	1	7	286.78	39.93	Susie	2	16	263.02	63.36
Susie	1	8	303.82	44.90	Susie	2	19	224.48	73.58
Susie	1	9	.	.	Susie	2	22	.	.
Susie	1	10	311.66	40.50	Susie	2	23	198.83	246.49
Susie	1	12	306.46	40.63	Susie	2	24	200.73	245.68
Susie	1	14	278.22	35.57	Susie	2	25	172.00	204.87
Susie	1	16	162.34	31.48	Susie	2	27	152.99	193.09
Susie	1	19	269.96	30.29	Susie	2	30	110.91	151.79
Susie	1	22	255.74	544.49	Susie	2	35	107.01	94.55
Susie	1	23	.	.	Susie	2	40	89.59	56.07
Susie	1	24	.	.	Susie	2	50	.	15.17
Susie	1	25	300.71	258.13	Susie	2	60	.	10.21
Susie	1	27	277.39	201.34	Susie	2	70	.	6.78
Susie	1	30	268.37	.	Susie	2	80	.	3.33
Susie	1	35	247.17	119.91	Susie	2	90	.	3.08
Susie	1	40	.	.	Susie	2	100	.	2.35
Susie	1	50	202.73	69.90	Susie	2	120	78.74	1.98
Susie	1	60	201.88	43.40	Susie	2	150	92.35	3.17
Susie	1	70	157.00	46.50	Susie	2	180	112.73	2.24
Susie	1	80	145.49	43.93	Sweetie	1	-30	135.95	6.68

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Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)	Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)
Susie	1	90	154.40	32.30	Sweetie	1	0	136.04	7.67
Susie	1	100	141.79	26.92	Sweetie	1	1	334.92	58.87
Susie	1	120	114.86	21.93	Sweetie	1	2	332.40	62.43
Susie	1	150	.	.	Sweetie	1	3	305.52	60.07
Susie	1	180	.	12.74	Sweetie	1	4	311.66	60.22
Susie	1	240	74.85	2.49	Sweetie	1	5	302.00	62.74
Susie	2	-30	113.92	5.47	Sweetie	1	6	344.73	57.02
Susie	2	0	106.18	4.68	Sweetie	1	7	310.00	66.02
Susie	2	1	307.40	43.35	Sweetie	1	8	337.66	50.02
Susie	2	2	311.16	45.94	Sweetie	1	9	.	.
Sweetie	1	10	309.75	46.88	Sweetie	2	4	347.50	23.53
Sweetie	1	12	298.57	44.56	Sweetie	2	5	313.90	.
Sweetie	1	14	287.51	37.31	Sweetie	2	6	336.69	19.10
Sweetie	1	16	321.13	45.16	Sweetie	2	7	326.22	20.44
Sweetie	1	19	286.93	.	Sweetie	2	8	268.01	21.20
Sweetie	1	22	290.88	527.60	Sweetie	2	9	335.11	22.79
Sweetie	1	23	252.36	400.93	Sweetie	2	10	297.31	20.20
Sweetie	1	24	298.98	313.89	Sweetie	2	12	311.83	16.68
Sweetie	1	25	270.15	255.29	Sweetie	2	14	287.89	17.06
Sweetie	1	27	293.41	242.63	Sweetie	2	16	293.32	.
Sweetie	1	30	250.15	193.69	Sweetie	2	19	321.14	18.09
Sweetie	1	35	302.59	140.69	Sweetie	2	22	320.03	206.32
Sweetie	1	40	277.03	115.78	Sweetie	2	23	247.99	194.44
Sweetie	1	50	242.02	99.41	Sweetie	2	24	233.56	99.58
Sweetie	1	60	252.28	65.98	Sweetie	2	25	259.53	143.98
Sweetie	1	70	247.55	68.87	Sweetie	2	27	284.15	107.95
Sweetie	1	80	261.34	69.03	Sweetie	2	30	282.03	89.04
Sweetie	1	90	233.25	53.76	Sweetie	2	24	233.56	99.58
Sweetie	1	100	176.97	53.63	Sweetie	2	25	259.53	143.98
Sweetie	1	120	230.25	62.01	Sweetie	2	27	284.15	107.95
Sweetie	1	150	144.10	33.95	Sweetie	2	30	282.03	89.04
Sweetie	1	180	134.94	20.29	Sweetie	2	35	231.00	53.53
Sweetie	1	240	114.65	65.82	Sweetie	2	40	227.79	42.53
Sweetie	2	-30	159.92	5.26	Sweetie	2	50	199.06	26.83
Sweetie	2	0	133.16	6.05	Sweetie	2	60	173.27	20.51
Sweetie	2	1	360.98	27.47	Sweetie	2	70	160.61	3.07
Sweetie	2	2	332.12	21.87	Sweetie	2	80	146.96	9.83
Sweetie	2	3	266.49	224.58	Sweetie	2	90	153.03	16.37

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Horse	FSIGT	Time point	Glucose (mg/dL)	Insulin (mU/L)
Sweetie	2	100	144.65	13.23
Sweetie	2	120	116.26	12.02
Sweetie	2	150	117.14	6.55
Sweetie	2	180	119.19	3.14

APPENDIX E

Horse	Day	Percent Weight Loss	Change in BCS	Lipolysis Media NEFA (mg/mL)	Lipolysis Media FG (mg/mL)
Boots	0	3.30	0	0.790	0.021
Boots	14	0.00	0	0.720	0.006
Boots	28	5.27	0	0.782	0.005
Boots	42	5.80	0	0.730	0.008
Boots	70		-1.5	0.796	0.014
Boots	98	12.50	-3	0.701	0.010
Daphne	0	-0.42	0	.	.
Daphne	14	0.00	0	1.291	0.029
Daphne	28	0.21	0	0.693	0.007
Daphne	42	1.69	0	0.755	0.009
Daphne	70		-1	0.630	0.014
Daphne	98	4.44	-3	0.676	0.008
Getda	0	-0.87	0	0.884	0.009
Getda	14	0.00	0	0.823	0.029
Getda	28	1.67	-0.5	0.764	0.031
Getda	42	2.78	-0.5	0.700	0.007
Getda	70		-2.5	0.641	0.009
Getda	98	6.83	-3	0.586	0.011
JR	0	-3.16	1	0.672	0.032
JR	14	0.00	0	0.813	0.017
JR	28	2.98	-0.5	0.626	0.005
JR	42	4.04	-1	0.575	0.015
JR	70	7.37	-2	0.714	0.006
Pistol	0	-1.78	-0.5	0.738	0.007
Pistol	14	0.00	0	0.816	0.012
Pistol	28	-1.13	0	0.762	0.020
Pistol	42	-0.47	0	0.805	0.005
Pistol	70		-1	0.606	0.011
Pistol	98	2.91	-2.5	0.651	0.015
Romeo	0	0.85	0	0.976	0.017
Romeo	14	0.00	0	0.732	0.013
Romeo	28	3.20	-0.5	0.981	0.017
Romeo	42	3.66	-0.5	0.829	0.010
Romeo	70		-2.5	0.852	0.011
Romeo	98	5.88	-3	0.735	0.007

Horse	Day	Percent Weight Loss	Change in BCS	Lipolysis Media NEFA (mg/mL)	Lipolysis Media FG (mg/mL)
Susie	0	-0.76	0	.	.
Susie	14	0.00	0	0.884	0.009
Susie	28	0.08	-0.5	0.677	0.008
Susie	42	1.43	-0.5	0.537	0.009
Susie	70		-1	.	.
Susie	98	5.63	-2.5	0.640	0.017
Sweetie	0	-0.74	0	1.171	0.033
Sweetie	14	0.00	0	0.762	0.007
Sweetie	28	4.26	-0.5	0.666	0.007
Sweetie	42	3.97	-0.5	0.657	0.012
Sweetie	70		-1.5	0.751	0.013
Sweetie	98	21.76	-3	0.703	0.010

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APPENDIX F

Horse	Day	Subpopulation	Complex I	Complex III	Complex IV
Boots	0	IFM	67.80	443.94	183.49
Boots	14	IFM	57.13	280.52	245.19
Boots	28	IFM	77.62	59.29	145.69
Boots	42	IFM	56.90	58.21	139.71
Boots	70	IFM	56.86	62.05	68.25
Boots	98	IFM	102.00	55.65	84.59
Boots	0	SSM	19.40	60.47	34.47
Boots	14	SSM	16.63	136.14	71.88
Boots	28	SSM	21.38	69.98	74.18
Boots	42	SSM	28.85	23.62	94.46
Boots	70	SSM	43.31	15.19	44.56
Boots	98	SSM	37.05	15.16	87.93
Daphne	0	IFM	37.00	226.44	72.67
Daphne	14	IFM	31.98	73.28	113.06
Daphne	28	IFM	74.34	89.66	117.84
Daphne	42	IFM	48.52	49.64	115.15
Daphne	70	IFM	31.95	12.07	94.95
Daphne	98	IFM	42.70	25.41	58.45
Daphne	0	SSM	68.57	67.82	52.38
Daphne	14	SSM	.	.	.
Daphne	28	SSM	46.06	99.78	47.01
Daphne	42	SSM	45.37	49.51	77.24
Daphne	70	SSM	49.92	9.90	61.41
Daphne	98	SSM	20.24	11.05	50.37
Getda	0	IFM	62.34	209.73	151.92
Getda	14	IFM	72.31	114.28	102.86
Getda	28	IFM	102.55	95.92	206.22
Getda	42	IFM	18.52	20.21	53.91
Getda	70	IFM	22.05	31.59	108.30
Getda	98	IFM	36.95	36.29	58.06
Getda	0	SSM	83.09	149.61	52.59
Getda	14	SSM	34.57	78.34	41.78
Getda	28	SSM	49.56	117.18	72.11
Getda	42	SSM	58.66	10.67	64.01
Getda	70	SSM	39.34	18.94	84.85
Getda	98	SSM	19.58	11.45	50.36

Horse	Day	Subpopulation	Complex I	Complex III	Complex IV
JR	0	IFM	54.15	166.18	163.97
JR	14	IFM	88.62	99.25	61.08
JR	28	IFM	49.95	81.76	110.37
JR	42	IFM	44.45	62.36	62.36
JR	70	IFM	49.82	58.25	65.24
JR	0	SSM	47.52	77.78	68.15
JR	14	SSM	32.85	132.35	92.64
JR	28	SSM	55.48	45.41	60.08
JR	42	SSM	50.58	34.50	115.91
JR	70	SSM	55.62	32.13	74.97
Pistol	0	IFM	31.90	174.07	85.87
Pistol	14	IFM	167.61	108.87	83.61
Pistol	28	IFM	83.45	150.25	234.94
Pistol	42	IFM	54.19	40.32	129.02
Pistol	70	IFM	34.57	21.22	101.85
Pistol	98	IFM	50.58	68.99	137.98
Pistol	0	SSM	45.12	45.22	22.31
Pistol	14	SSM	27.09	147.81	65.04
Pistol	28	SSM	39.20	133.66	72.71
Pistol	42	SSM	17.85	8.35	18.37
Pistol	70	SSM	14.17	11.60	57.98
Pistol	98	SSM	21.72	16.16	53.01
Romeo	0	IFM	41.07	82.16	137.43
Romeo	14	IFM	78.85	109.71	144.55
Romeo	28	IFM	.	.	159.95
Romeo	42	IFM	37.15	23.39	76.71
Romeo	70	IFM	.	20.64	80.49
Romeo	98	IFM	33.08	23.20	4.64
Romeo	0	SSM	37.80	45.21	39.02
Romeo	14	SSM	39.74	140.12	104.09
Romeo	28	SSM	44.51	.	60.93
Romeo	42	SSM	19.95	14.51	56.59
Romeo	70	SSM	44.66	33.74	94.48
Romeo	98	SSM	38.76	23.79	65.02
Susie	0	IFM	18.53	64.46	62.18
Susie	14	IFM	43.58	103.76	103.76
Susie	28	IFM	.	.	155.22
Susie	42	IFM	37.20	22.14	64.21

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1880

Horse	Day	Subpopulation	Complex I	Complex III	Complex IV
Susie	70	IFM	34.64	37.81	109.64
Susie	98	IFM	28.54	26.69	26.69
Susie	0	SSM	28.05	52.48	85.28
Susie	14	SSM	55.61	75.86	64.73
Susie	28	SSM	40.96	52.15	137.07
Susie	42	SSM	42.08	9.84	58.06
Susie	70	SSM	28.48	19.98	53.28
Susie	98	SSM	23.18	12.65	40.48
Sweetie	0	IFM	26.15	61.82	70.38
Sweetie	14	IFM	65.68	107.51	225.78
Sweetie	28	IFM	42.84	105.18	154.26
Sweetie	42	IFM	73.28	66.64	175.93
Sweetie	70	IFM	64.51	44.00	144.31
Sweetie	98	IFM	18.48	45.38	.
Sweetie	0	SSM	26.54	100.48	67.35
Sweetie	14	SSM	37.74	103.90	69.64
Sweetie	28	SSM	19.70	89.32	60.54
Sweetie	42	SSM	28.90	47.31	87.52
Sweetie	70	SSM	32.68	35.66	87.97
Sweetie	98	SSM	9.43	15.44	30.88

1881

1882

Horse	Day	Subpopulation	ATP Synthase	Citrate Synthase	Lipid Peroxidation
Boots	0	IFM	1.406	113.12	0.1527
Boots	14	IFM	3.948	63.68	0.3232
Boots	28	IFM	2.414	76.84	0.1750
Boots	42	IFM	.	47.99	0.1868
Boots	70	IFM	4.421	19.84	0.1073
Boots	98	IFM	2.115	51.23	0.1578
Boots	0	SSM	2.442	306.17	.
Boots	14	SSM	2.328	282.26	0.4316
Boots	28	SSM	1.994	325.64	.
Boots	42	SSM	1.683	154.15	0.3476
Boots	70	SSM	1.684	220.59	0.4313
Boots	98	SSM	2.881	294.64	0.3793
Daphne	0	IFM	2.501	238.78	0.7276
Daphne	14	IFM	1.492	97.84	0.7900
Daphne	28	IFM	1.825	110.57	0.3792
Daphne	42	IFM	1.886	33.86	0.2320
Daphne	70	IFM	0.764	265.58	0.4550
Daphne	98	IFM	1.207	94.69	0.3296
Daphne	0	SSM	1.111	273.64	0.6772
Daphne	14	SSM	.	.	.
Daphne	28	SSM	1.264	383.69	0.4165
Daphne	42	SSM	0.941	141.09	0.4906
Daphne	70	SSM	2.117	408.31	0.9560
Daphne	98	SSM	1.259	378.65	.
Getda	0	IFM	1.616	151.49	0.8532
Getda	14	IFM	2.714	186.71	0.7077
Getda	28	IFM	2.278	87.91	0.6325
Getda	42	IFM	0.960	129.68	0.2622
Getda	70	IFM	1.286	172.08	0.2638
Getda	98	IFM	1.724	143.35	.
Getda	0	SSM	1.938	529.52	0.5126
Getda	14	SSM	1.240	503.41	0.3804
Getda	28	SSM	2.997	225.83	0.8267
Getda	42	SSM	1.520	373.12	0.5553
Getda	70	SSM	2.159	235.05	0.5131
Getda	98	SSM	2.175	531.35	0.7145

Horse	Day	Subpopulation	ATP Synthase	Citrate Synthase	Lipid Peroxidation
JR	0	IFM	2.105	49.45	.
JR	14	IFM	2.176	35.91	0.2424
JR	28	IFM	0.971	30.30	0.1102
JR	42	IFM	4.937	21.69	0.1907
JR	70	IFM	1.107	35.89	0.0683
JR	0	SSM	1.407	150.90	0.6942
JR	14	SSM	2.357	187.61	0.5711
JR	28	SSM	3.982	185.10	0.3491
JR	42	SSM	1.311	99.81	0.2718
JR	70	SSM	2.544	128.76	0.2675
Pistol	0	IFM	1.654	36.95	0.0949
Pistol	14	IFM	4.551	64.51	0.0967
Pistol	28	IFM	2.595	52.89	0.1442
Pistol	42	IFM	1.532	60.13	0.3293
Pistol	70	IFM	0.672	46.33	0.0459
Pistol	98	IFM	3.933	37.44	0.0821
Pistol	0	SSM	2.291	243.48	0.8462
Pistol	14	SSM	2.808	264.03	0.4042
Pistol	28	SSM	4.571	173.59	0.2529
Pistol	42	SSM	1.454	230.30	0.5857
Pistol	70	SSM	1.377	168.69	0.3518
Pistol	98	SSM	0.921	263.28	0.2470
Romeo	0	IFM	2.129	66.75	.
Romeo	14	IFM	8.583	93.45	0.5432
Romeo	28	IFM	2.235	85.66	0.3866
Romeo	42	IFM	0.889	89.45	0.4396
Romeo	70	IFM	0.980	137.63	0.1774
Romeo	98	IFM	2.572	85.41	0.3240
Romeo	0	SSM	2.939	267.18	.
Romeo	14	SSM	4.754	229.50	0.1669
Romeo	28	SSM	2.831	183.31	0.4122
Romeo	42	SSM	3.446	221.28	0.4324
Romeo	70	SSM	3.205	219.82	0.3687
Romeo	98	SSM	1.130	292.59	0.3497
Susie	0	IFM	1.081	89.38	0.3483
Susie	14	IFM	1.232	61.69	0.2953
Susie	28	IFM	2.633	40.14	0.3365
Susie	42	IFM	1.578	89.71	0.1587

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Horse	Day	Subpopulation	ATP Synthase	Citrate Synthase	Lipid Peroxidation
Susie	70	IFM	1.796	61.81	0.3624
Susie	98	IFM	1.268	86.16	0.2618
Susie	0	SSM	1.870	195.80	0.5381
Susie	14	SSM	1.441	334.50	0.4622
Susie	28	SSM	2.123	122.79	0.3825
Susie	42	SSM	1.870	373.73	0.3802
Susie	70	SSM	1.582	297.03	.
Susie	98	SSM	1.803	300.94	0.4682
Sweetie	0	IFM	3.162	31.57	0.5239
Sweetie	14	IFM	3.830	50.87	0.2193
Sweetie	28	IFM	2.498	63.08	0.4065
Sweetie	42	IFM	1.266	42.02	0.1979
Sweetie	70	IFM	1.672	53.64	0.1215
Sweetie	98	IFM	1.437	40.63	0.0817
Sweetie	0	SSM	1.548	254.97	0.7668
Sweetie	14	SSM	1.067	298.52	0.4283
Sweetie	28	SSM	0.943	269.80	0.2072
Sweetie	42	SSM	1.685	160.82	0.2615
Sweetie	70	SSM	4.517	162.81	.
Sweetie	98	SSM	2.054	136.21	0.4772

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